Effects of Gestational Nutrition on Post-Natal Fertility in Sheep

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Abstract

Changes to feed availability resulting from global climate change have the potential to exacerbate fertility issues already facing the NZ livestock industry. Appropriate feeding levels during gestation is gaining more attention as a number of studies have illustrated that underfeeding during gestation can have negative impacts on the fertility of female offspring. However, the mechanisms underlying this relationship remain obscure. Therefore, the aims of this study were to firstly establish a model in sheep whereby restricted gestational nutrition influenced fertility of the female offspring. A second aim was to identify potential mechanisms underlying the relationship between restricted gestational nutrition and postnatal fertility.

Ewes were provided with either a maintenance diet, or a 0.6 of maintenance diet for the first 55 days of gestation. Thereafter, all ewes were fed ad-lib for the remainder of gestation. Fetuses were collected at days 55 and 75 of gestation to examine fetal ovarian development using stereology, and RNAseq was used to examine gene expression. Steroid profiles were generated from both maternal and fetal (day 75 only) plasma samples. Female offspring were monitored from birth until 19 months of age. From these offspring, the time of onset of puberty was recorded, indicators of fertility (ovulation rate and antral follicle counts) were assessed at 8 and 19 months of age, and key hormone profiles were generated at 19 months of age.

Surprisingly, female offspring at 19 months of age, but not 8 months of age, showed increases in key indicators of fertility: ovulation rate ($p < 0.05$) and antral follicle count (AFC, $p < 0.01$). Additionally, these animals showed an increase in plasma progesterone concentrations ($p < 0.05$) indicative of increased embryo survival. Changes to the pattern of FSH secretion ($p < 0.05$) were also observed.

Fetal ovaries exposed to restricted nutrition contained more germ cells at day 75 but not at day 55 of gestation ($p < 0.01$). RNAseq identified 69 sequences differentially expressed in fetal ovaries at day 55, and 145 sequences at day 75. Fold changes observed at day 75 were less than those observed at day 55. Amongst differentially expressed genes, germ cell specific genes were prominent at both ages. Prominent Gene Ontology categories at both ages were ion transport and protease inhibitors. Pathways identified as affected using IPA analysis included some related to the metabolism of arginine to nitric oxide and citrulline, LXR/RXR and FXR/RXR activation, quantity of germ cells, GADD45 signalling, and acute phase response signalling.

Taken together, the data supports increased indicators of fertility in female offspring whose dams were exposed to restricted nutrition during gestation. The observed differences appear to originate within the ovary. The results are consistent with the concept that it is not the restricted nutrition alone, but the change in nutrition from restricted to ad-lib which may be generating the observed changes in the offspring. Further, the data offers insights into potential mechanisms underlying the phenotype observed with both nitric oxide and protease inhibitors being possible candidates for involvement.

The results open new avenues of research to either address current fertility issues in livestock, or to improve livestock fertility through manipulating gestational nutrition.

Acknowledgments

While working with sheep is a joy, it has its challenges: a long estrus cycle, a long gestation, and a long time to reach reproductive maturity. This made the project a busy, three and a bit year adventure. The time spent with animals creates a special bond between researchers and the animals, and therefore the first acknowledgement must go to those animals who sacrificed so much, and were such fun to work with.

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As Laurel also happens to be my wife, her contribution has extended beyond the lab. I thank her for her patience and her contributions to long whiteboard discussions, planning sessions over a Pinot Gris (or two), and help with editing of this thesis. Long has she suffered the absences from home life, the forgetfulness of everyday things while the brain focused on genes and hormones, the frustrations, and the excited narratives in the good times. We did survive.

To my committee members Greg Anderson and Peter Hurst, a thank you for keeping me on track and on time. Peter especially has been a long-time colleague and mentor; his thoughts and inputs have been greatly appreciated. Thanks to AgResearch, (particularly Sara Edwards and

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To my supervisors, Jo-Ann Stanton and Jenny Juengel, what can I say? I know I have presented you two with some special challenges at times. However, through your knowledge, patience and perseverance you have seen me through, kept me focused, and taught me so much, not just about the science, but also about planning, writing, and philosophy… Okams razor will never be forgotten.

Finally, I would like to dedicate this thesis to my late mother and father. They always had faith in my ability, even if I did not. It may have taken 30 years to get around to this, but it is done. I hope I have made you both proud.

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List of Abbreviations

°C	degrees Celsius
µm	micrometre
µmol/L	micromoles per litre
1°Ab	primary antibody
2°Ab	secondary antibody
Ad lib	ad libitum
AFC	antral follicle count
AMH	anti Mullerian hormone
ANOVA	analysis of variance
BAM	binary alignment map
BCS	body condition score
BOC	bovine ovine caprine
bp	base pairs
BrdU	5bromo 2deoxy-uridine
cDNA	complimentary deoxy ribonucleic acid
CIDR	controlled intra-uterine releasing device
CL	corpus luteum
cm	centimetre
CV	coefficient of variation
DAB	diamino benzidine
DEV	deviation from expected value
DNA	deoxy ribonucleic acid
dUTP	2'-deoxyuridine 5'-triphosphate
ED	effective dose
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ERCC	External RNA Control Consortium
FDR	false discovery rate
fpkm	fragments per kilobase of exon, per million reads
FSH	follicle stimulating hormone
g	relative centrifugal force

g	gram
g/L	grams per litre
GC	guanine cytosine
GLM	general linear model
GnRH	gonadotrophin releasing hormone
GO	gene ontology
GREL	gonadal ridge epithelial like cells
H&E	haematoxylin and eosin
HRP	horse radish peroxidase
IU	international units
I^{125}	radioactive isoform of iodine
IgG	immuno globulin G
IHC	immunohistochemistry
IPA	Ingenuity Pathway Analysis
ISP	ion sphere particle
kg	kilogram
Ki-67	proliferation marker
LCMS	liquid chromatography mass spectroscopy
LH	luteinising hormone
M	maintenance
MAPLC3	microtubule associated protein light chain 3
ME	metabolisable energy
mg	milligram
MJ	mega joule
MJME	mega joules of metabolisable energy
mL	millilitre
mM	milli molar
mmol/L	millimoles per litre
mRNA	messenger ribonucleic acid
n	number (refers to sample size)
ng/mL	nanograms per millilitre
NIDDK	National Institute of Diabetes and Digestive and Kidney Diseases

NRS	normal rabbit serum
NSB	non-specific binding
NZ	New Zealand
OAR3	ovis aries 3 genomic database
OD	optical density
OR	ovulation rate
PARP	poly(ADPribose) polymerase
PCOS	poly cystic ovary syndrome
PCR	polymerase chain reaction
pg/mL	picograms per millilitre
pmol/L	pico moles per litre
PMSG	pregnant mares serum gonadotrophin
QC	quality control
qRT-PCR	quantitative real time PCR
R	restricted
RAR	retinoic acid receptor
RARE	retinoic acid response element
RIA	radioimmunoassay
RIN	referential integrity number
RMANCOVA	repeated measures analysis of covariance
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
ROS	reactive oxygen species
RT	room temperature
RXR	nuclear retinoid x receptor
SAM	sequence alignment map
SAR	sheep anti-rabbit
SD	standard deviation
SHRP	streptavidin horse radish peroxidase
TGFβ	transforming growth factor beta
TUNEL	terminal deoxynucleotidyl transferase

Chapter 1 . Introduction

The Barker hypothesis was first proposed in 1990 (*1*), and describes a relationship between intra-uterine growth retardation and the prevalence of disease in the offspring. Since this time, interest in the developmental origins of health and disease has grown. Links between maternal environment and postnatal hypertension (*2*), heart disease (*3, 4*), obesity (*5*), diabetes (*6*), lung disease (*7*), and fertility (*8*), have been described. The relationship between maternal environment and postnatal fertility first gained attention through studies of women born during the Dutch famine in the later stages of World War II (*9*). In addition to possible implications for human health, the effects of environment (such as maternal nutrition) on lifetime fertility has implications for the livestock industry. The NZ livestock industry faces significant fertility challenges, particularly for beef and dairy farming. Changes to feed availability resulting from global climate change has the potential to contribute further to fertility issues already facing the livestock industry. The issue of feed availability during pregnancy has become increasingly important as a number of studies have shown that restricted maternal nutrition can affect fetal ovarian development, and subsequently, negatively affect the fertility of female offspring (*10, 11*). While the relationship between maternal under-nutrition and decreased fertility in female offspring is gaining increasing attention, the underlying mechanisms behind this effect are yet to be clearly established. The development of practicable strategies to overcome this effect would benefit from a greater understanding as to how maternal under-nutrition can impact fertility in the offspring.

The overall aim of this study was to use sheep as a model to examine the underlying mechanisms by which maternal under-nutrition may affect fetal ovarian development and subsequently fertility in female offspring. As this study involves aspects of fetal development, as well as adult fertility, this introduction will firstly outline fetal, and fetal ovarian development, with a particular emphasis on the sheep. Secondly, adult fertility in the sheep will be outlined, with particular emphasis on ovarian follicle development and function. Finally, some relevant publications examining maternal nutrition and postnatal fertility will be discussed.

1.1 Fetal ovarian development

Gestation in the sheep spans 147 days and can be divided into three equal trimesters (*12*). During the first trimester, both embryonic/fetal growth and metabolic activity are high, however, as the embryo is small, maternal energy requirements are relatively low (*13*). During the second trimester, fetal development is relatively low, while growth and differentiation of the placenta remains high. Finally, during the last trimester, fetal growth is rapid and maternal nutritional demand is high. The implication is that as maternal energy requirements increase throughout gestation, nutrition restriction later in gestation is more likely to produce effects on the offspring, at least with regard to overall fetal growth.

Critical to the development and growth of the fetus is the placenta. This organ is responsible for the exchange of nutrients and waste products between the maternal and fetal circulatory systems (*14*). In the sheep, placental development begins during the final stages of implantation, between days 16 and 22 of gestation (*15*). Following implantation, the placenta undergoes exponential growth until around day 75 of gestation, but slows there-after (*16*). Most studies suggest that during the first trimester the placenta is able to adapt to restricted nutrition so that, providing normal nutrition is restored, fetal nutrition and growth is maintained during late gestation (*17, 18*). Conversely, nutrition restriction in mid to late gestation alters placental development and leads to intra-uterine growth restriction (*16, 19*). Interestingly, in adolescent sheep, over-feeding also reduces placental development around mid-gestation, leading to intra uterine growth restriction in late gestation (*20*). Overfeeding resulted in changes to nutrient partitioning, promoting rapid growth of the adolescent animal (particularly the deposition of fat) at the expense the placenta (*21*). The consensus view of the literature is consistent with the view that under-nutrition during the mid to late stages of gestation is more likely to affect overall fetal growth (and subsequently birth weight) than under-nutrition during the early stages of gestation (*12, 22*).

Fetal organ development occurs simultaneously with the period of exponential placental growth (days 22 to 75). As the developmental pattern of each organ differs, susceptibility to maternal under-nutrition may vary between organs (*23*). Pillai and colleagues demonstrated in sheep that altered maternal nutrition, even for a short period early in gestation, can affect organ development (*24*). In the Pillai study, underfeeding between days 30 to 45 of gestation resulted in increased liver size at day 45, while overfeeding between days 30 to 45 resulted in increased kidney size at day 45 (*24*). Thus, while altered maternal nutrition during early gestation seems less likely to affect overall fetal growth, effects on individual organs have been demonstrated.

The developing ovary (or gonadal ridge) is first observed around day 22 in the sheep as a thickening of the coelomic epithelium on the medial aspect of the mesonephros (*25*). Primordial germ cells migrate to, and populate the gonadal ridges. This migration is partially regulated by the cytokine, stem cell factor (*26*), as well as extracellular matrix proteins, particularly fibronectin (*27*). At these early stages, the developing ovary consists of primordial germ cells, invading mesonephric stromal cells, and gonadal ridge epithelial like cells (GREL) derived from the surface epithelium of the mesonephros (*28*). Invasion or migration of mesonephric cells into the developing ovary continues until around day 90 in the sheep. These mesonephric cells are initially a loose arrangement of mesenchymal cells (*29*) and/or cells of the giant nephron (*30*), but subsequently these become a discrete continuum of cells which are thought to develop in to the ovarian rete (*31*).

Morphological sexual differentiation occurs around day 32 in the sheep. A number of factors are essential for the differentiation of the ovary including β catenin, follistatin, FOXL2, R-respondin, and Wnt4 (*32*). In contrast to the testis, the ovary develops into two zones. The medulla in the central area, and an outer cortical region. From the time of sexual differentiation until at least day 45, the ovary is capable of producing increasing amounts of oestradiol (*33*). Within the ovary, germ cells located in the cortex form close associations (desmosomes) with GREL cells. GREL cells have a distinctive gene expression profile (*28*) and ultrastructure (*34*), and are thought to be the precursors of granulosa cells. Following sexual differentiation, in addition to germ cells and GREL cells, the ovary also consists of mesenchymal cells, endothelial cells of the developing vasculature, and cells at the ovarian surface (referred to as superficial GREL cells) (*28*). From its first appearance until approximately day 90 of gestation, the ovary lacks a true surface epithelium as a continuous underlying basement membrane is absent (*28, 34*). Throughout this period (day 22 to day 90), germ cells continue to proliferate.

The process of ovigerous cord development defines the ovarian cortex (which contains the germ cells and ovigerous cords) from the medulla, and occurs between days 38 and 90 in the sheep. Ovigerous cord development and subsequent dissolution is outlined in Figure 1.1. This process is first characterised by the development of cytoplasmic extensions from the GREL cells that isolate individual germ cells, and the increasing appearance of basal lamina material adjacent to the germ cells. By day 55, germ cells and pre-granulosa cells are confined within a distinct membrane enclosed tube or cord. The cords extend from the innermost cortical regions radially out towards the ovarian surface where they are open ended. Penetration of stromal cells into the ovarian cortex further defines the appearance of the ovigerous cords.

Historically the formation of cords in developing rodent ovaries has not been well described, prompting debate in relation to potential developmental differences between rodent and sheep. In the mouse, cord formation is sometimes overlooked or confused with the formation of clusters (nests) of germ cells. The development of these nests has been well illustrated in a number of publications (*32*, *35*). The developmental pattern in mouse is clearly outlined by Sarraj and Drummond (*36*) and is strikingly similar to sheep, with clusters of mitotic germ cells forming between days 10.5 to 13.5 post conception, followed by formation of ovigerous cords containing meiotic germ cells from day 14.5 post conception. Cords and nests both appear to isolate oocytes and pre-granulosa cells from the surrounding stroma. The absence of germ cell nest formation (as described in the mouse) from sheep development may represent a subtly different pattern of development between rodents and ruminants. It is thought this difference reflects differences in the time period between sexual differentiation and the onset of meiosis (*37*).

During the development of ovigerous cords, germ cell numbers in the fetal sheep ovary increase substantially from 50,000 at day 40, to reach peak numbers of approximately 1 million at day 75 (*38*). Further, it is estimated that over the same period, pre-granulosa cell numbers increase from 200,000 to 4.2 million (*39*). Each germ cell is in direct contact with pre-granulosa cells, yet BrdU labelling suggests that while germ cell proliferation is high, proliferation of pre-granulosa cells within the cords is very low (*34*). Given that the germ cells are isolated from the surrounding stroma by a basement membrane, it has been postulated that further recruitment of pre-granulosa cells occurs from GREL derived cells near the ovarian surface where the cords are open. Arguably this then represents two waves of pre-granulosa cell recruitment in the fetal sheep ovary, one before cord formation, and one after. Such a scenario is strikingly similar to the two waves of recruitment proposed in the mouse by Mork and colleagues (*40*).

From approximately day 55, the key germ cell developmental processes of meiosis and apoptosis are evident (*41*), with formation of follicles appearing from day 75 (*38*). Within the cords, a gradient of germ cell development is observed, with the more developed cells located in the innermost regions of the cortex, and the lesser developed germ cells located towards the periphery. This pattern is observed both morphologically for meiosis and follicle formation, and also in the pattern of gene expression, notably expression of *POU5F* and *DAZL* (*28*).

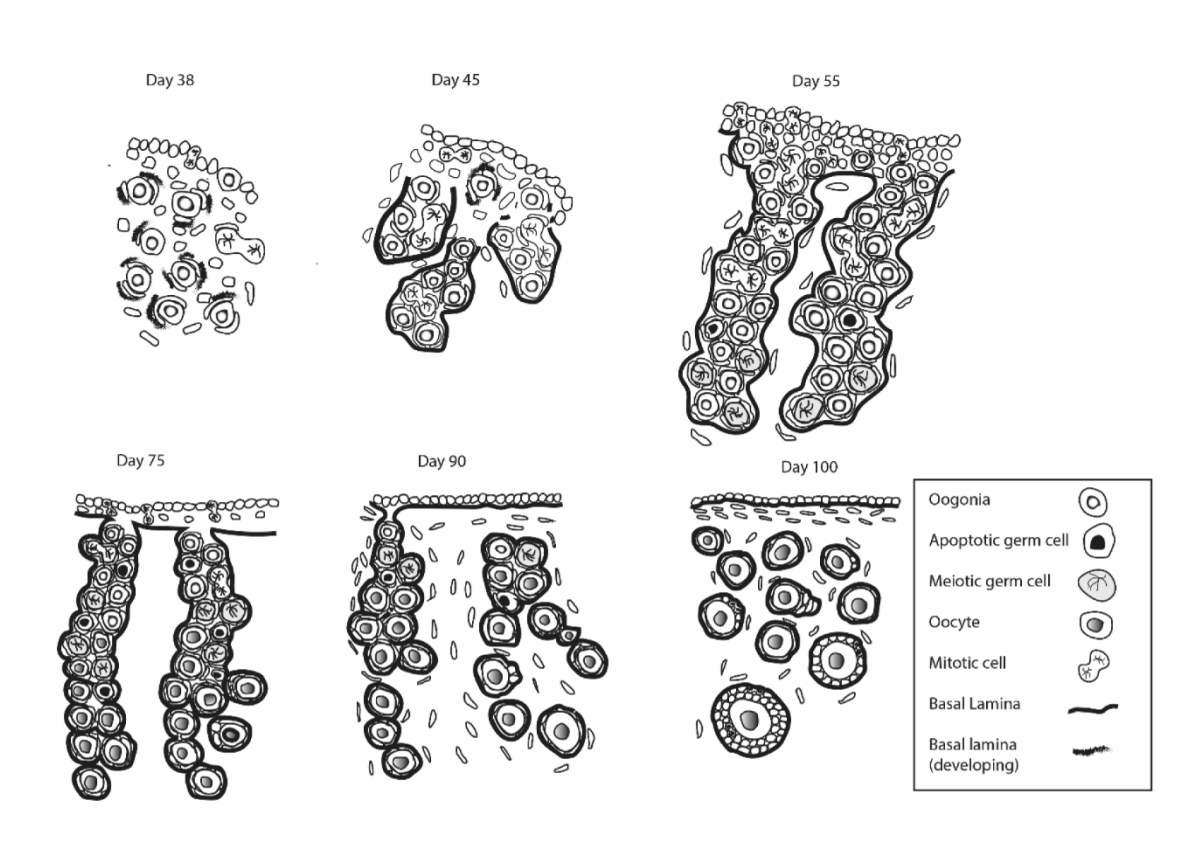

Figure 1.1 Schematic illustration of ovigerous cord development. At day 38, proliferating germ cells are in direct contact with pre-granulosa cells. By day 45, extensive deposits of basal lamina are observed around oogonia. By day 55, ovigerous cords are well established but remain open to the ovarian surface. Within the cords, mitosis, meiosis and apoptosis of germ cells is evident and these continue to be observed at day 75. At day 75, the first follicles are forming at the base of the cords. By day 90, most germ cells are arrested in prophase I of meiosis and cords become separated from the ovarian surface epithelium, which now rests on a discrete basement membrane. By day 100, most germ cells are present in isolated follicles and the first growing follicles are evident. Modified with permission from Juengel et al (*29*).

Meiosis largely occurs between days 55 and 90 of gestation. The initiation of meiosis is widely thought to be regulated by retinoic acid (*42*). Retinoic acid acts by diffusing through tissues and binding to nuclear receptors (RARs) that heterodimerise with nuclear retinoid X receptors (RXRs). The dimers bind to retinoic acid response elements (RARES) in the promoter region of target genes and thereby control the regulation of retinoic responsive genes (*43*). In the fetal mouse ovary, retinoic acid is thought to be sourced from the mesonephros (*44*). In humans, retinoic acid produced by the fetal ovary is thought to initiate meiosis (*45*). The source of retinoic acid in the fetal sheep ovary has not been studied. However, the migration of a discrete continuum of cells from the mesonephros to the ovary, coinciding with the initiation of meiosis (*34*), would suggest that this continuum of cells may be a source of ovarian retinoic acid. Meiosis is a tightly regulated process, with retinoic acid produced by the *ALDH1A* genes, and metabolised by *CYP26B1* (*46*). Expression levels of these genes play a pivotal role in the regulation of meiosis. A number of other genes are critical for successful meiosis, these include *DMC1, POU5F1, STRA8,* and *SYCP3* (*47*).

Increased evidence of germ cell death is observed coinciding with the onset of meiosis. Death of germ cells in fetal sheep ovaries becomes especially prevalent between days 75 and 90 of gestation. During this interval, germ cell numbers fall from approximately 1 million to 120,000 (*38*). This precipitous decline in germ cell numbers is a common feature during development in most mammals. As germ cells undergo cell death, it is evident that their associated pre-granulosa cells are not destined to the same fate, but are “reassigned” to adjacent germ cells (*34*, *48*). In mice, germ cell loss occurs in two waves, one wave during meiosis, and one wave during follicle formation (*36*).

Despite recent evidence implicating a role for autophagy in the death of germ cells (*49*), the consensus is that most germ cells die via an apoptotic pathway (*50*). Apoptosis is largely regulated by members of the BCL-2 family, consisting of the anti-apoptotic factors BCL-X, BCL-2 and MCL-1, as well as the pro-apoptotic factors BID, BAX, BOK and BAD. The relative expression of these factors contribute to the level of apoptosis (*51*). Also critical in apoptosis are caspases and their target poly(ADPribose) polymerase (PARP), with both of these factors used as markers of early apoptosis (*49*). Caspases are cysteine proteases that execute cell death, either as initiators of apoptosis, effectors of apoptosis, or acting through cytokine activation. Initiator caspases are activators of the effector caspases which, upon activation, execute the apoptotic process. Using immunoblotting, Fulton and colleagues demonstrated the presence of the initiator caspases 8 and 9, effector caspases 2, 3 and 7, and PARP in human

fetal ovaries at the time of peak germ cell apoptosis (*48*). The same study also showed activated caspase 3 localised to germ cells undergoing apoptosis (*48*), thus highlighting the importance of these factors in gem cell apoptosis.

From approximately day 75 of gestation in the sheep, ovarian follicles can be observed at the base of the ovigerous cords (*52*). There are two proposed mechanisms by which isolated follicles arise. Hummitzsch and colleagues (*28*) proposes an active role of the ovarian stroma in penetrating ovigerous cords, dividing the cords into smaller nests and subsequently isolating individual follicles. Juengel and Smith(*25*), and Sawyer and colleagues (*34*) propose increasing secretion of basal lamina material by the germ cell - pre-granulosa cell complexes, causing dissolution of the cords leading to isolated follicles. It is plausible that both mechanisms play a role in the formation of isolated follicles.

By approximately day 90 of gestation, the first growing follicles are observed, and by day 120 antral follicles are present. The mechanisms by which resting follicles are recruited into the growing pool of follicles are not clearly understood. Thecal cells can be morphologically identified surrounding follicles early in their growth. The origin of theca cells has been debated for some time. As early as 1991, Hirshfield proposed that thecal cells were associated with follicles at the time of initiation of growth (*53*). Young and McNeilly proposed follicle driven differentiation of stromal cells into thecal cells (*54*). Honda and colleagues proposed a population of thecal stem cells (*55*), while Hatzirodos and colleagues reported factors associated with stem cell niches in the theca externa (*56*). Juengel and colleagues proposed that mesonephric derived cell streams are likely sources of theca cells (*29*). In a recent study, Liu and colleagues proposed a dual source of thecal cells (*57*), *WT-1* positive cells intrinsic to the ovary, and *GLI-1* positive cells derived from the mesonephros. While still the subject of debate, the Liu hypothesis appears to include elements from most of the hypotheses listed above. Liu and colleagues propose populations of pre-determined cells, some of mesonephric origin, being driven to differentiate into thecal cells by signals from the oocyte and granulosa cells.

Figure 1.2 summarises the timing and duration of the key developmental steps during fetal ovarian development in the sheep, and compares this to human development. In both sheep and human, all the key developmental processes occur before term (147 days for sheep and 280 days for humans). The similarities in the pattern of development between species suggests that the sheep may be a good model for human ovarian development. The sheep is already a widely accepted model for studying Poly Cystic Ovarian Syndrome (PCOS) (*58*), a condition affecting 10% of women worldwide (*59*). The model uses prenatal exposure to androgens to produce changes in fetal ovarian development, leading to female offspring displaying symptoms resembling PCOS in humans (*60*).

While there is the potential for the hypothesised germ line stem cells to make an ongoing contribution to the ovarian reserve (*61, 62*), it is widely accepted that following follicle formation, germ cell and follicle numbers (the ovarian reserve) are already established for an individual (rodents being an exception, where ovarian development continues into the postnatal period). Therefore, in sheep, as with most mammals, the ovarian reserve is set before birth (*39*). The size of the ovarian reserve then has a significant impact on the individual's fertility in postnatal life (*63-65*).

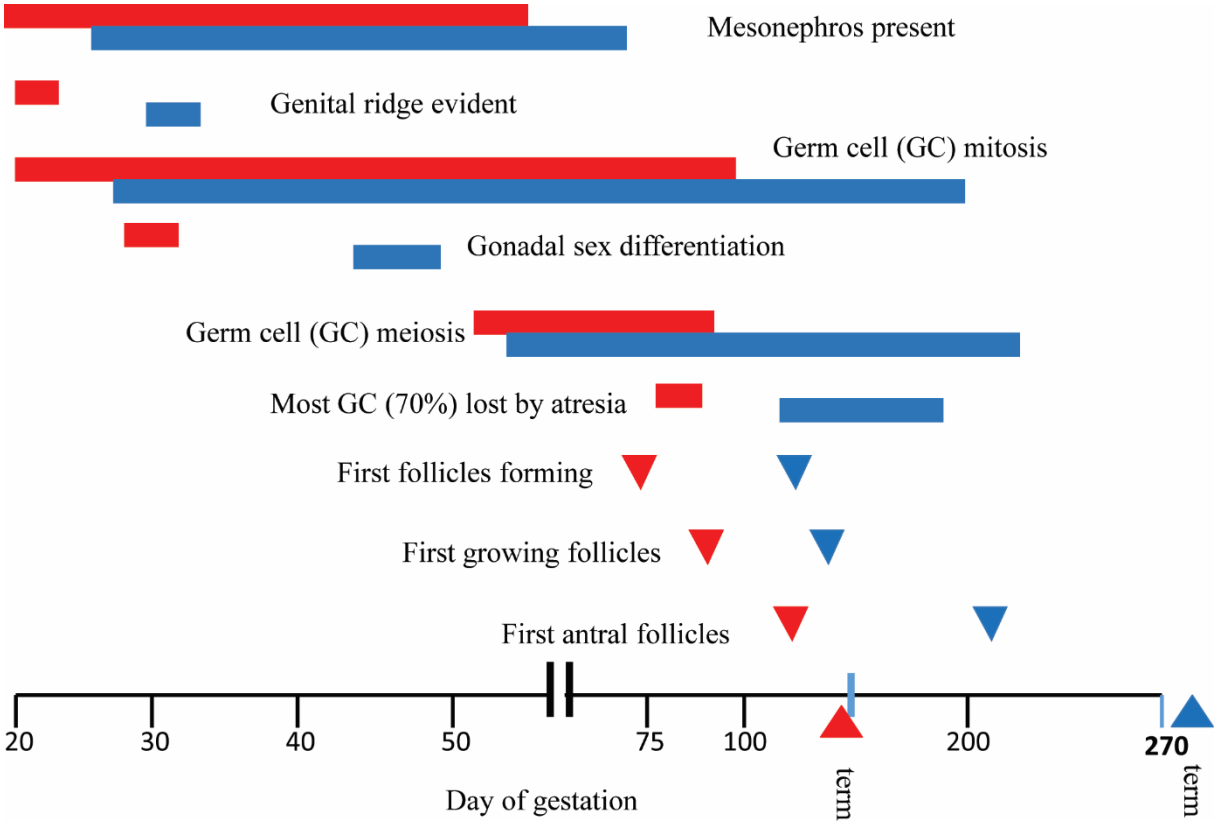

Figure 1.2 Chronology of events during ovarian development. Comparison of the timing of the key developmental effects during fetal ovarian development in sheep (red bars and arrowheads) and humans (blue bars and arrowheads), with both species showing a similar pattern in the timing of these events. Data compiled from (*39*).

1.2 Postnatal fertility in the female sheep

Sheep are seasonal breeders. In the New Zealand environment females display oestrous activity usually between March and July, although wide variations are apparent depending on the breed of the animal and geographical location (latitude). Lambs are usually born between August and November, with most animals attaining puberty by April or May of the following year, at 8 to 9 months of age (*66*).

The initiation of puberty in sheep is a complex process and not fully understood. In sheep, typical of mammals, body weight and the level of fat deposition is critical in determining puberty onset. The consensus proposes that blood borne factor(s) reflecting the metabolic status of an animal affect the neuroendocrine system ultimately resulting in increased gonadotrophin secretion and triggering puberty. Various factors have been implicated in this process including glucose (*67*), amino acids (*68*), free fatty acids (*69*), insulin (*70*), insulin like growth factor 1 (IGF-1) (*71*) and leptin (*72*).

Leptin is primarily produced in adipose tissue but its production has been observed in other tissues including the pituitary (*73*) and the placenta (*74*). In the context of this thesis, the relationship between nutrition, leptin (commonly described as the satiety hormone), puberty, and fertility makes this hormone worth discussing.

The effects of leptin on puberty in rodents have been well studied but results vary. Leptin administered to immature mice accelerated the onset of puberty (*75, 76*). In rats administration of leptin did not advance puberty initiation but did negate the effects of food restriction on the timing of puberty (*77*), with the authors concluding that leptin was not the primary signal but one of a number of permissive factors for initiation of puberty (i.e. a threshold level is essential to allow the onset of puberty).

In cattle, Zieba and colleagues (*78*) found that body weight was the largest contributing factor accounting for variations in age at puberty onset and this was highly correlated with serum leptin levels. However unlike the mouse, administration of leptin did not advance the onset of puberty (*79, 80*).

Mutations in the leptin receptor gene have been identified in sheep which are associated with differences in the timing of puberty onset (*81*) as well as decreases in fertility in these animals (*82*), suggesting a role for leptin not only in the onset of puberty but also in fertility. However by contrast, Recabarren and colleagues (*83*) showed that maintenance feeding of prepubertal female sheep for 10 weeks when compared to ad libitum feeding initially reduced leptin concentrations. At the completion of the 10 week experiment, leptin concentrations in the maintenance group were comparable to those in the ad libitum group. While the onset of puberty was not reported in this study, the authors concluded from their data that leptin concentrations may not be associated with the onset of puberty.

While the studies reported above appear somewhat contradictory, it appears that, in sheep, leptin has a role to play, but is likely to be one of a number of signals regulating puberty. The control of diets (restricted, maintenance and ad libitum) in these studies, as well as the metabolic state of the animals during the trials, may also account for some of the variation in the effects seen. Further, species differences may also account for some of the variation in the importance of leptin demonstrated in these studies, particularly given that some animals are seasonal breeders while others are capable of breeding all year round, some are polyovular while others are not, sheep and cattle are ruminants as opposed to monogastric rodents and humans.

The reproductive performance of sheep mated at 8 to 9 months of age (having just attained puberty) is lower than that observed in adults. The poor reproductive performance in these animals is due to lower ovulation rates, failure to mate, failure of fertilisation, and increased embryo loss (*84*). Why peri-pubertal animals have lower fertility is currently the subject of several studies. One potential clue comes from the study of Mork and colleagues, which describes two waves of pre-granulosa cell recruitment in mice (*40*). Follicle dynamics (growth rates) in prepubertal mice are different from adults (*85*), with this difference being linked to the recruitment of granulosa cells during fetal development. During the prepuberty period, growing follicles contain granulosa cells derived from the first wave of follicle recruitment, while postpubertal follicles contain granulosa cells derived from the second wave of recruitment. Prepubertal follicles grow substantially faster (*86*), display a different morphology (*85*), and show a different expression pattern of steroidogenic genes (*87*) when compared to postpubertal follicles. Should these findings in mice translate to sheep, the differences in granulosa cell origin and follicle dynamics between prepubertal and postpubertal animals may account for the differences in fertility observed between pubertal and mature sheep. The concept of multiple waves of granulosa cell recruitment also highlights the importance of cells, other than germ cells, during ovarian development to postnatal fertility.

Reproduction in sheep, as with most mammals, is regulated by the hypothalamic-pituitary-gonadal (HPG) axis (Figure 1.3). Gonadotrophin releasing hormone (GnRH) produced by the hypothalamus stimulates pituitary production of both luteinising hormone (LH) and follicle stimulating hormone (FSH) (*88*). All three hormones are secreted in a pulsatile manner. FSH and LH act on the ovary to support follicle growth (FSH) and induce final follicle maturation and ovulation (LH) (*89*).

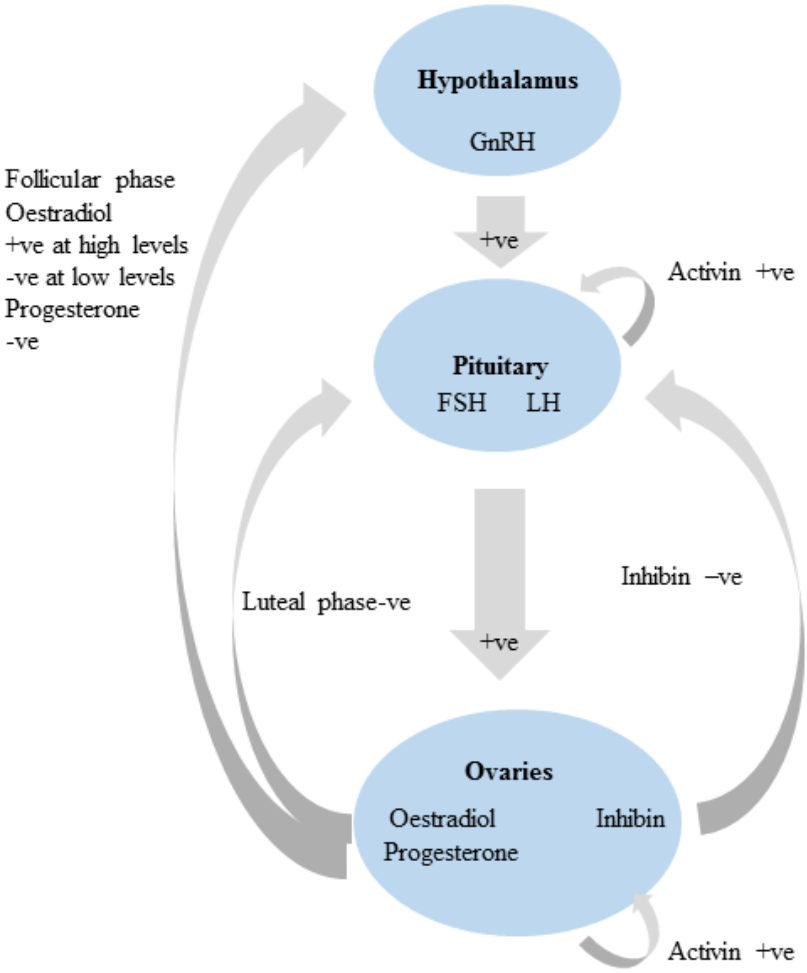

Figure 1.3 Regulation of sheep reproduction. Pulsatile GnRH produced by the hypothalamus stimulates pulsatile secretion of FSH and LH from the pituitary. FSH stimulates follicle growth, while LH stimulates final follicle maturation and ovulation. Ovarian steroids provide negative feedback on the pituitary, but high concentrations of oestradiol during the follicular phase provides positive feedback on the hypothalamus to stimulate the pre-ovulatory gonadotrophin surge. Throughout the cycle, ovarian produced inhibin provides negative feedback on the pituitary, while activin, produced in the pituitary and ovary, acts in a paracrine manner stimulating both pituitary and ovarian function. Increasing levels of oestradiol from the pre-ovulatory follicle drive simultaneous peaks of FSH and LH which result in ovulation. Figure compiled from (*90-93*).

The emergence of each follicle wave is accompanied by a transient increase in FSH concentration (*94*). Administration of exogenous FSH results in the emergence of an additional follicle wave (*95*). These studies conclude that the emergence of each follicle wave is driven by FSH. While oestradiol and inhibin produced by the follicle are known to affect FSH secretion, their importance in regulating the rhythmical pattern of FSH secretion throughout the ooestrous cycle is unclear. Baby and Bartlewski highlighted the potential for progesterone to influence the pattern of FSH secretion and the number of follicle waves within a cycle (*94*), concluding however, that more work is required to clarify the factors involved in the establishment of follicle waves. However, Duggavathi et al (*96*) demonstrated a continued rhythmical pattern of FSH secretion in ovarectomised ewes, concluding, at least in part, an ovarian independent rhythmical pattern of FSH secretion.

As described in Section 1.1, in sheep, as with most mammals, the ovarian reserve is set during fetal life. Several studies have established concepts that highlight the importance of the ovarian reserve and its relationship to fertility. Firstly, the establishment of the ovarian reserve is highly variable between individual animals, and appears to establish the relative fertility of the individual (*63, 97*). This variability is consistent with the concept that establishment of the ovarian reserve may be influenced by maternal environment. The impact of maternal environment on ovarian reserve has been demonstrated with maternal nutrition (*65*) and maternal disease (*98*), both being shown to influence the size of the ovarian reserve. Secondly, ovaries with a high ovarian reserve have a high antral follicle count (*64, 97*) (the number of growing follicles present within the ovary, referred to as AFC). Thirdly, individuals with a low AFC show low fertility compared to individuals with a high AFC (*63, 97*) . This evidence is consistent with a strong link between the ovarian reserve, AFC, and fertility. However, Monniaux and colleagues contend that fertility is compatible with a range of AFC values, and that the number of ovulations characteristic of each species does not depend on the reserve of growing follicles (*64*). Their conclusion however, does not preclude within-species variations in fertility dependent on ovarian reserve or numbers of growing follicles.

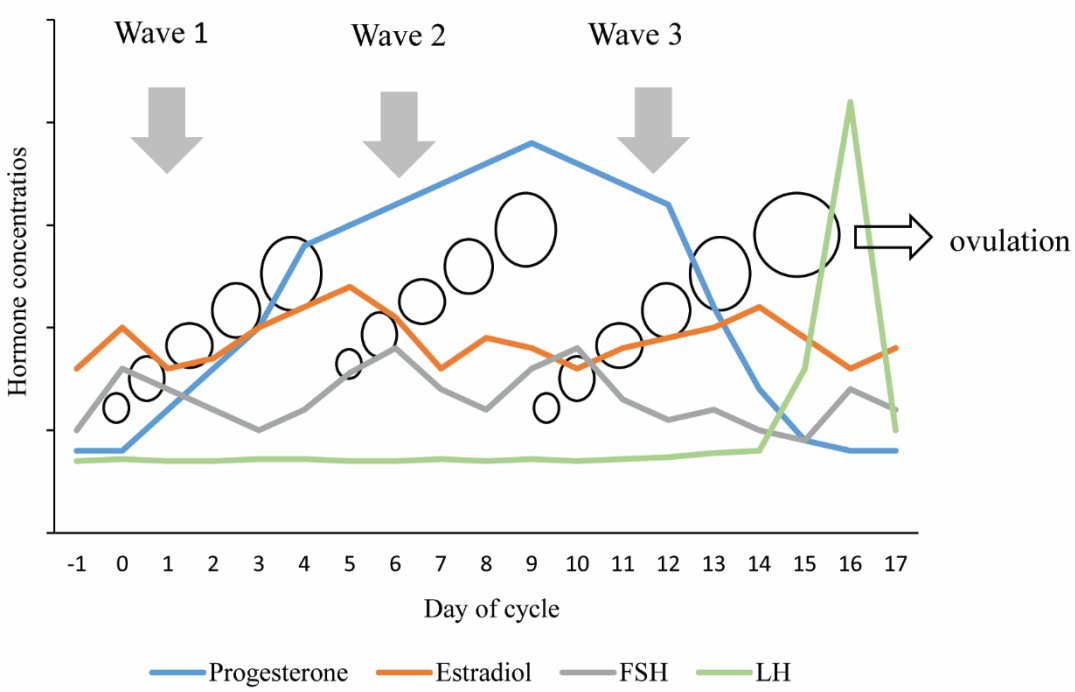

Figure 1.4 Characteristics of the sheep oestrous cycle. Patterns of hormone secretion throughout the estrous cycle in relation to the pattern of emergence of three waves of follicles, indicated by the arrows. Follicles are indicated by black circles. Throughout each wave, the majority of follicles are lost to atresia. Data compiled from Baby and Bartlewski (*94*).

Follicles are continually recruited from the non-growing pool until the reserve becomes depleted to such an extent that reproduction declines substantially (as in sheep) (*99*), or ceases (as in humans). Follicles develop through a number of well-defined developmental stages (Figure 1.5) as they progress to ovulation. However, most follicles do not reach the ovulatory stage but die through a process of atresia at various stages of development. Non-growing follicles (primordial follicles) are characterised by an oocyte surrounded by a single layer of flattened granulosa cells (type 1, Figure 1.5). The first sign of follicle growth is the transformation of some cells from flattened to cuboidal in shape (transitional or type 1a, Figure 1.5). While this classification system is widely accepted and applied to most mammalian species, it is known that at least in sheep, some follicles are formed with a mixture of flattened and cuboidal granulosa cells (*25*). Whether these follicles are actually growing is not known. Thereafter, growth involves proliferation of granulosa cells, the establishment of multiple concentric rings of granulosa cells, and the development of a fluid filled antral space (type 5, Figure 1.5). During follicle development, the oocyte increases in size from 35 µm to around 120 µm (*100*).

Growth factors and hormones are produced by the follicles and act in both an endocrine and paracrine manner. Follicles between 0.1 and 3 mm in diameter are often classified as gonadotrophin responsive (type 3, 4 and 5. Figure 1.5), where exposure to gonadotrophins, particularly FSH, will elicit a cellular response (*101*). In the absence of gonadotrophins, follicle growth still continues up to approximately 3 mm (*85, 102*). At approximately 3 mm in diameter (late type 5), follicles are classified as gonadotrophin dependent. In the absence of gonadotrophins, follicle growth does not proceed beyond this stage (*102*). Factors produced by the ovary which include progesterone, oestradiol, and inhibin, in turn feed-back to act on the pituitary and hypothalamus to regulate secretion of both LH and FSH (*91, 103*). Additionally, pituitary produced activin acts in a paracrine manner to increase gonadotrophin secretion (*92*). The process of follicle development, along with the factors and their receptors, either produced by or present in the developing follicles, are outlined in Figure 1.5. This is not an exhaustive list, but highlights some of the major factors/receptors available to act on the follicle.

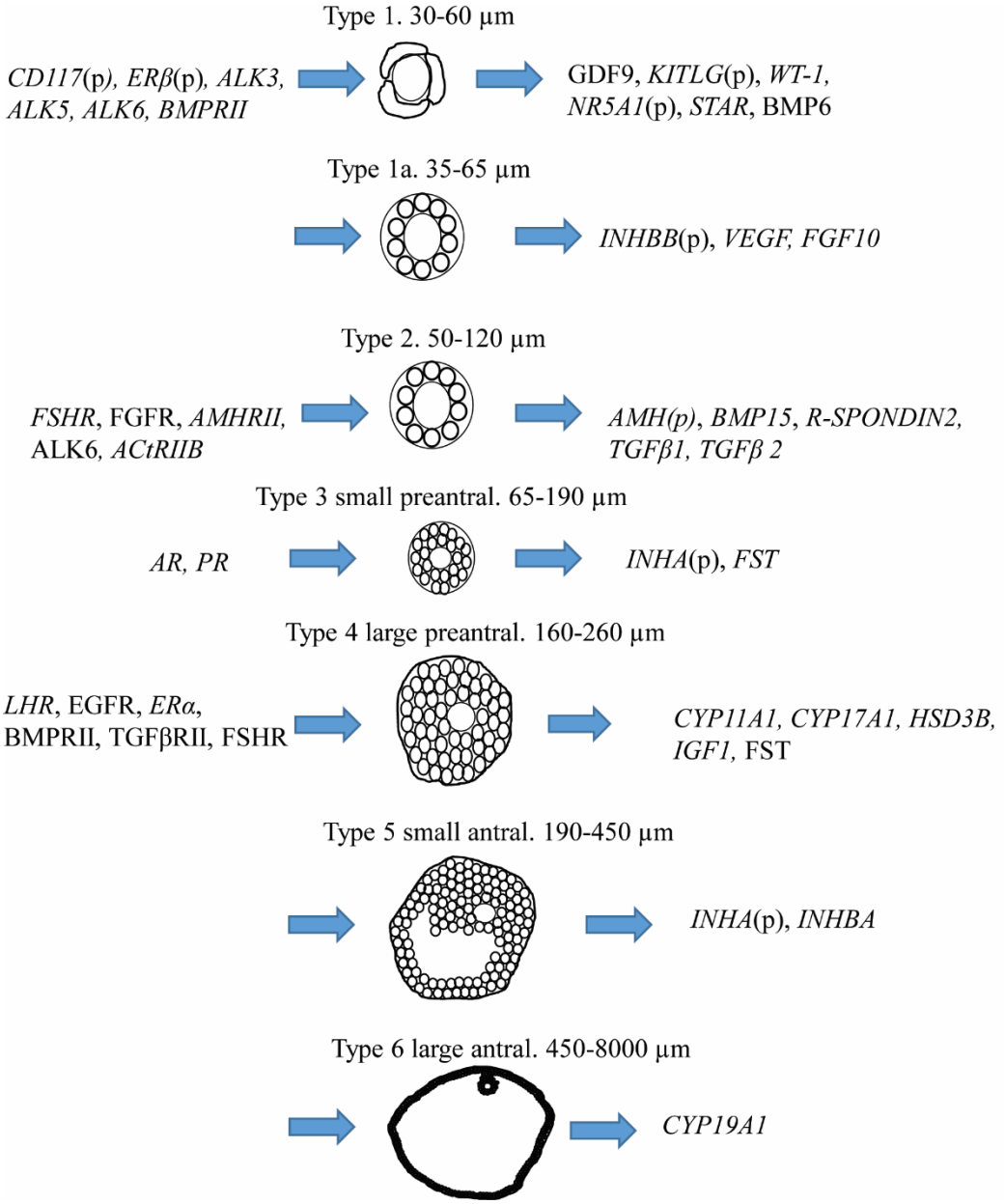

Figure 1.5 Morphological and functional development of ovarian follicles. Beginning with type 1 non-growing follicles up to 60 µm, follicles progress through developmental stages. Initial growth is characterised by a change in shape of follicular granulosa cells, then proliferation of the granulosa cells which form concentric rings. From type 5, a fluid filled antrum develops. Most growing follicles die through atresia at various developmental stages. Throughout the process, genes (in italics) controlling growth factors (right hand side) and receptors (left hand side) are activated, and the presence of growth factor and receptor proteins (regular font) can be detected. (p) following italicised items indicates both gene and protein have been detected. The figure has been compiled across a number of mammalian species from the following references (*104-117*).

Discussion of all the hormones and factors regulating reproduction and ovarian development is beyond the scope of this literature review. However, some of the factors potentially relevant to this study known to be involved in ovarian/follicle development (inhibins, activins, AMH, steroids) along with leptin which is involved in both nutrition and reproduction, will be discussed.

Inhibins and Activins. Inhibins and activins are members of the TGFβ superfamily. Both inhibins and activins are dimers comprised of two similar subunits (*118*). The structure of activins and inhibins is shown in Figure 1.6. Genes encoding inhibin and activin subunits are expressed during follicle development (*108, 109*). During fetal development, expression of both inhibin α and inhibin βA subunit are detected in the sheep fetal ovary only late in gestation once growing follicles are present (*119, 120*). Expression of type 1 and type 2 receptors has also been observed in developing follicles (*121*). Activins interact with two classes of receptors (type 1/ALK receptors, and type 2 receptors) (*103*). Inhibin signalling is somewhat more complex, but involves binding to ACTR2. While the structure of activins and inhibins is similar, their functions differ markedly. Inhibins suppress synthesis and secretion of FSH, whereas activins enhance FSH synthesis and secretion.

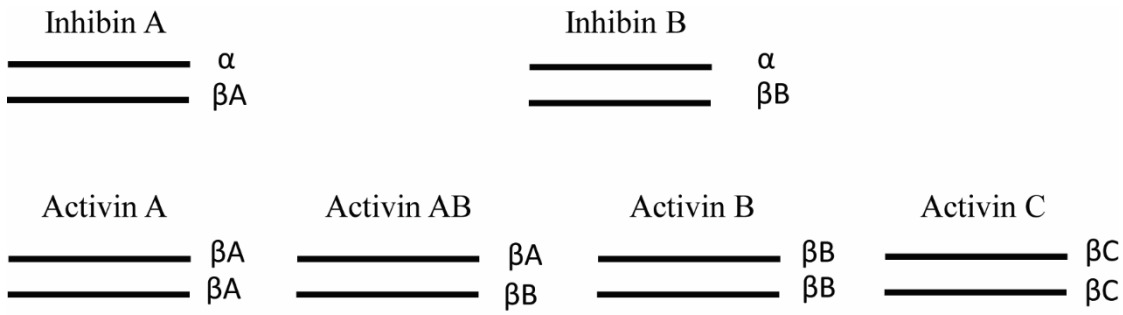

Figure 1.6 Structure of activins and inhibins. Activins and inhibins are proteins. Each is a dimer comprising of two related inhibin subunits represented by the black lines (either inhibin α, inhibin βA, inhibin βB or inhibin βC) linked by disulphide bonds. Each subunit is derived from a specific gene. The subunit composition determines the identity and function of the protein. Figure compiled from (*92*).

Both inhibins and activins are produced by follicular granulosa cells and have been associated with a number of functions within the ovary including cell proliferation, gonadotrophin receptor regulation, and steroid synthesis (*122*). While fetal ovarian production of inhibins and activins

is not apparent in the sheep fetal ovary until late in gestation, inhibins and activins have been found in gonadal extracts and amniotic fluid from sheep fetuses of both sexes (*123*). Whether these levels are due to ovarian production is unclear, as the placenta also produces increasing amounts of activins and inhibins from as early as the first trimester (*124*). However, in the primate, Billiar and colleagues proposed an important role for activins and inhibins in development (*125*). They showed an association between decreased numbers of follicles and increased expression of the α inhibin subunit and suggested the ratio of inhibins:activins was critical for development. In the human fetal ovary, activin A has been implicated in regulating germ cell proliferation (*121*).

Anti Mullerian hormone (AMH). AMH is also a member of the TGFβ superfamily and was originally described as the molecule responsible for the regression of the mullerian ducts in the male fetus (*126*). AMH acts through two receptors, AMHR2 and a type 1 receptor which has yet to be identified with any certainty. While there is some doubt about the type 1 receptor, Belville and colleagues have implicated ALK3 as a likely candidate (*127*). In the adult female, *AMH* is expressed in the granulosa cells of follicles from the type 2 to 3 follicle stage of development (*128*). Expression is greatest in follicles at antrum formation and declines thereafter, with this decline being correlated with increased *CYP19A1* expression (*129*). The expression pattern of *AMHR2* is similar to that for *AMH* (*115*). In the rodent, AMH is thought to be an important regulator of the initiation of primordial follicle growth and later follicle development (*130*). However, this does not appear to be the case in sheep. In sheep, AMH appears to regulate the number of follicles transitioning from the gonadotrophin responsive phase to the gonadotrophin dependent phase (*129*). Of greatest relevance to this study is the use of AMH as an indicator of fertility. High plasma concentrations of AMH have been associated with high AFC, high ovarian reserve and high fertility in a number of species including human, rodents, cow, and sheep (*63, 131, 132*). This relationship is now widely accepted, and AMH concentrations are the basis of a number of diagnostics tests to assess fertility in humans (*133, 134*).

Steroids. Ovarian steroid hormones can be assigned to three types: oestrogens (e.g. oestradiol, estrone), androgens (e.g. androstenedione, testosterone) and progestogens (e.g. progesterone). Each of these classes of steroids play a critical role in ovarian function, follicle development, and development of the fetal ovary. In the mature ovary, androstenedione produced by follicular thecal cells, serves as a substrate for oestrogen synthesis by granulosa cells from the same follicle (*135, 136*). Oestrogen is critical for development of the follicle to the ovulatory stage,

and regulation of the gonadotrophin hormones FSH and LH (*137, 138*). Additionally, both androgens and oestrogens play a role in apoptosis of follicular granulosa cells, with androgens enhancing apoptosis, and oestrogens inhibiting apoptosis (*136, 139*). Oestrogen has also been demonstrated to play a role in establishing the size of the ovarian reserve in mice (*140*). Progesterone is produced by the corpus luteum following ovulation, and its key roles are the establishment and maintenance of pregnancy (*15*). A number of studies have demonstrated a relationship between increased progesterone concentrations and increased embryo survival (*141-143*).

In the fetus, while the placenta is a major site of steroid production, the fetal ovary can produce steroids during early gestation (*33, 144*). Oestrogens, androgens and progesterone have all been demonstrated to have significant impacts on fetal ovarian development, particularly in follicle formation and germ cell development (*145, 146*).

Leptin. Leptin is primarily an adipocyte secreted hormone important in maintaining energy homeostasis. Leptin is highly responsive to nutrition and is critical in the control of body weight (*147*). The genes responsible for leptin production and its receptor are also expressed in a wide range of tissues including the ovary (*148*), pituitary (*149*), hypothalamus (*150*), as well as the fetus and placenta (*151*). In the brain, leptin has been found to facilitate GnRH secretion from the hypothalamus (*152*) and is thought to stimulate both LH and FSH secretion from the pituitary (*153*). Further, these actions potentially occur via a nitrous oxide pathway. In the rat ovary, exogenous leptin has been shown to decrease ovulation rate (*154*). However, in the sheep, increased endogenous levels of leptin increases ovulation rate (*155, 156*). While not essential for pregnancy, leptin is thought to play an important role in nutrition during gestation (*157*), and appears important in the onset of puberty and potentially fertility as discussed earlier.

1.3 Gestational nutrition and postnatal fertility

Studies examining the effects of gestational nutrition on fertility vary markedly in both the methods used and the results. In addition to the species studied, variables to be considered include: the type of diet employed (metabolisable energy, calorie restriction, protein restriction), the amount of dietary restriction (or amount of overfeeding), control diet (maintenance or ad libitum), and the gestational period over which dietary changes are applied. In an approach aimed at overcoming these variations, Asmad (*158*) used meta-analysis on thirteen separate studies to examine the effect of maternal nutrition on reproductive

development. This analysis showed no effect of prenatal nutrition on postnatal reproductive performance based on ovarian weight, follicle counts and LH concentrations. Asmad (*159*) does point out that the analysis used a small number of studies, and that the variables analysed may not truly indicate reproductive performance.

However, numerous studies have shown that alterations to gestational nutrition can affect fetal ovarian development and/or postnatal reproductive performance (Table 1.1). While not all studies affect the fertility of the offspring, it would appear that under the right experimental conditions, postnatal fertility can be impacted.

There are two likely mechanisms by which gestational nutrition may affect postnatal fertility. The first mechanism is via an epigenetic mechanism (*160*). Epigenetics involves changing the structure of DNA (and consequently gene expression) without altering the DNA sequence. Epigenetics predominantly involves DNA methylation, histone modification, or binding of small RNAs to DNA (*161*). A well-studied example linking epigenetic changes to ovarian development and function is the prenatal exposure to environmental oestrogens, recently reviewed by Cruz and colleagues (*162*). In rats, exposure to methoxychlor (MXC, an endocrine disrupting chemical used in pesticides) between day 19 of gestation and day 7 postnatal, resulted in increased expression levels of DNA methyltransferases. One result of this increased expression was hyper methylation of the promoter region of the oestrogen receptor gene ERβ, leading to ovarian dysfunction in the adult rats (*163*). Lea and colleagues demonstrated in sheep that maternal exposure to endocrine disrupting chemicals (EDCs) contained in sewage sludge altered the fetal transcriptome. Amongst the genes differentially regulated following exposure to EDCs were genes coding for histone and DNA methylation, consistent with EDCs triggering altered states mediated by epigenetic mechanisms (*164*). In sheep, prenatal exposure to bisphenol A (BPA, used in the manufacture of plastics) is known to cause disruptions to the ovarian cycle (*165*). Veiga-Lopez and colleagues showed in sheep that prenatal exposure to BPA altered the transcriptome of the fetal ovary (*166*). Expression of miRNAs targeting the *SOX* family of genes, kit ligand, and insulin related genes (all important in ovarian development) were affected by BPA exposure. While not definitive in its conclusions, the Veiga-Lopez study does nonetheless highlight the potential for altered expression of miRNAs to affect postnatal ovarian function.

Table 1.1 Gestational nutrition and postnatal fertility, synopsis of relevant publications

Reference	Species	Diet	Duration of altered nutrition (days)	Fetal effect	Adult effect	Fertility of offspring
Long **(*167*)**	ovine	110% vs. 50%	28-78		↓progesterone	↓
Martin **(*168*)**	bovine	Protein supplementation	Late gestation		↑pregnancy rates Higher % calved	↑
Asmad **(*159*)**	ovine	Ad lib vs. maintenance	21-140	↑ type 1 & 2 follicle numbers at d100 and 140		
Bernal **(*169*)**	rat	Ad lib vs. 50%	Whole gestation		↓Pi, 2^{o}, antral follicle ↑oxidative stress	
Kotsampasi **(*170*)**	ovine	100% vs. 50%	0-30		↑FSH response to GnRH Smaller CLs Difference in follicle size distribution	
Lea **(*171*)**	ovine	100% vs. 50%	0-30 0-110 & 66-110	↓Ki-67at d65 ↑BAX at d110 ↑BAX and MCL-1		
Borwick **(*172*)**	ovine	150% vs. 50%	0-47 0-62	↑germ cells at d47 ↑germ cells at d62 ↑meiotic germ cells		

Reference	Species	Diet	Duration of altered nutrition (days)	Fetal effect	Adult effect	Fertility of offspring
Meikle **(*173*)**	mouse	Ad lib vs. food deprived for 4 out of 10 days	Variable		↓number of pups born from 2nd mating	↓
Costa **(*174*)**	bovine	180% vs 60%	0-60	↓ovarian wt at d60 Altered ovarian transcriptome at d60		
Da Silva **(*175*)**	ovine	High vs moderate	0-103	↑Pi follicles at d103 ↑ plasma progesterone		
Grazul-Bilska **(*176*)**	ovine	100% vs 60%	50-135	↓ovary weight ↓proliferation in Po follicles at d135		
Rae **(*177*)**	ovine	100% vs 50%	0-95		↓OR at 20 months	↓
Rae **(*178*)**	ovine	100% vs 50%	0-55 0-65	↓ ovarian weight at d55 ↓ oocytes at d65 ↓follicle development at d110		
Evans **(*179*)**	bovine	100% vs 60%	First trimester		↓AFC	

Diet column % relates to a maintenance diet, e.g. 150% is a diet equivalent to 150% of maintenance. ↓ indicates reduction in the lower diet group, ↑ indicates increase in the lower diet group. Pi = primordial follicles. Po = primary follicles. 2° = secondary follicles.

In most mammalian species, paternal genome demethylation occurs immediately after fertilisation, followed by remethylation around the blastocyst stage of development. However, the sheep appears to be an exception to this pattern (*180*). In sheep, demethylation of primordial germ cells occurs during their migration to the developing gonad (*181*). The timing of remethylation of germ cells is sex dependent. In the female germ cell, remethylation is a gradual process and occurs once the germ cells are arrested in meiosis I (*181*). In the developing sheep ovary, this would begin from approximately day 55 of gestation. Alterations to gestational nutrition can potentially enhance variations in this epigenetic reprogramming process, leading to permanent changes in phenotype which can persist into the F1 (somatic cells and germ cells affected) and F2 (germ cells only affected) generations (*182, 183*).

Alternatively, nutrition induced changes may be developmental only. Differences in nutrient availability may lead to temporal up or down regulation of gene expression. These changes may lead to structural alterations in fetal ovarian development while not necessarily affecting DNA. Consequently, non-heritable phenotypic differences in the offspring (F1 generation) may be apparent. For example, temporal changes to genes regulating germ cell proliferation or germ cell death may affect the ultimate size of the ovarian reserve and consequently the fertility of the offspring. However, without changes to the DNA (e.g. through methylation, or altered miRNA binding), any changes to fertility would not be passed to the F2 generation of animals.

1.4 Overview and aims

Results of numerous studies examining the effects of gestational nutrition vary depending on species, methodologies used, and the type and timing of the altered nutrition used. Despite these investigations, the underlying mechanisms whereby gestational nutrition affects both fetal development and postnatal animals have not been identified. Additionally, there is a lack of studies utilising the same cohort of animals to examine both fetal development and postnatal effects.

The sheep was selected as the model for the studies presented in this thesis for two reasons. Firstly, from an agricultural industry perspective, the sheep is an economically important animal. In 2015, Statistics New Zealand estimated the number of sheep in New Zealand at approximately thirty million, contributing over three billion dollars to the economy in 2014. The challenges facing the sheep industry (climate change and the use of increasingly marginal land) have implications for consistently providing adequate levels of nutrition, and therefore

maintaining the current levels of fertility. With reproductive patterns being similar between the major livestock species, the sheep is an ideal representative model for the livestock industry, as it is more cost effective and manageable from an experimental viewpoint. Fertility is a major challenge facing the livestock industry as genetic selection for economically valuable traits (e.g. milk production, carcass quality) have led to declining fertility, particularly in the beef and dairy industries.

Secondly, fetal ovarian development in the sheep is remarkably similar to humans, as outlined in Figure 1.2. While sheep are seasonal breeders, they are none the less a low ovulation rate species similar to human. Therefore, results from a trial utilising sheep may have the potential to be extrapolated to human biology.

From a livestock industry perspective, the long term goal of this project is to propose management strategies, and/or nutritional supplements during gestation to maximise the fertility of female offspring. However, before considering supplementation, a better understanding of the relationship between gestational nutrition and postnatal fertility is required.

Two hypotheses guided the work presented in this thesis.

Firstly, that restricted maternal nutrition will lead to changes in fetal ovarian gene expression and fetal ovarian morphology that have the potential to alter ovarian development.

Secondly, that restricted maternal nutrition will lead to reduced fertility in female offspring.

From these hypotheses the specific aims of this study were to:

- Establish a model in sheep whereby gestational under-nutrition during early gestation produced a measurable effect on postnatal fertility.
- Characterise measurable changes in the maternal environment resulting from reduced nutrition.
- Characterise the postnatal effects on the physiology and endocrinology of mature female offspring.
- Characterise the effects of gestational nutrition on the development of the fetal ovary, focusing on germ cell development and fetal ovarian gene expression.

Chapter 2 . Establishment and Validation of the Animal Model

2.1 Introduction

A primary goal of this study was to establish and describe a model in sheep where restricted gestational nutrition impacted postnatal female fertility. As highlighted in Section 1.3, many studies have reported that restricted gestational nutrition can negatively impact postnatal female fertility (*168, 173, 177*). While some studies report changes to fetal ovarian development (*176, 178, 184*), the mechanism(s) underlying the relationship between gestational nutrition and postnatal fertility have yet to be clearly identified. Therefore, in this work both plasma and tissue samples were collected for downstream applications to explore any effects observed, and to investigate potential mechanisms underlying these observations. These downstream applications are reported in Chapters 3, 4, 5 and 6.

Major considerations for the model were the degree and timing of nutrition restriction. Both were based on previous studies and met the guidelines laid out by the institutional animal ethics committee. Trials involving indoor restricted nutrition on pregnant ewes had not previously been undertaken by research groups within AgResearch. Additionally, trials involving fetal collections from large animals are now subject to stringent examination by AgResearch ethics committees. For this trial the ethics committee requested that both the period and degree of nutrition restriction be set at the minimum likely to produce an effect.

The timing of nutrition restriction was set for the first 55 days of gestation. This period was designed to encompass developmental processes that may be affected by nutrition. This included germ cell migration, gonadal sexual differentiation, germ cell proliferation, the early stages of meiosis, and germ cell apoptosis (*38*). This timing means that the restricted nutrition period does not fully cover other important developmental processes within the fetal ovary including meiosis (predominantly day 55-75), the peak wave of germ cell atresia (day 75-90) and follicle formation (day 75-100). Alterations to these key processes are likely to have major impacts on ovarian development and potentially postnatal ovarian function. However, previous studies in sheep using nutrition restriction early in gestation (prior to day 55) demonstrated alterations to fetal ovarian development (*171, 172*). Further Rae and colleagues contend that the period of maximum sensitivity of the sheep fetus to undernutrition lies between the day of mating and day 65 of gestation (*178*). Impacts in adult offspring from dams exposed to restricted nutrition early in gestation have not been fully examined to date.

The degree of nutrition restriction was set at 0.6 of a maintenance diet based on metabolisable energy intake (with control animals set at maintenance). This was considered the minimum degree of restriction likely to produce effects on fetal development and postnatal fertility. While a level of 0.5 of maintenance is commonly used in sheep (*171, 178*), 0.6 has been used previously (*176*), and is commonly used in cattle (*179*).

The selection of a maintenance diet, as opposed to an ad libitum diet, for the control group was based on reported effects of obesity and high nutrition on maternal environment, fetal growth and development, and effects on the offspring. An ad libitum diet is likely to result in over nutrition, substantial maternal weight gain and increased fetal fat deposition. In sheep maternal over nutrition has been shown to retard both placental and late fetal growth, as well as increasing fetal loss in late gestation (*21*). Further, feeding at 125% and 150% of maintenance to produce overweight and obese animals respectively, results in larger fetuses at mid-gestation (*185*). Maternal obesity from feeding at 150% of requirements has also been shown to produce multigenerational effects on the offspring including the failure to display an early postnatal peak in leptin, increased adiposity, and increased plasma levels of glucose, insulin, and cortisol. (*186*). Therefore to avoid the potential effects of over nutrition, a maintenance diet was employed for control animals.

The selection of days 55 and 75 to recover fetuses for subsequent studies was based on two factors. Firstly, in the context of this project, day 55 would allow determination of any effects of the complete period of restricted nutrition on both fetal development, and fetal ovarian development. Day 75 would give an indication as to the longevity of any changes noted at day 55. Secondly, considerable data on sheep ovarian development has been published at both day 55 and 75 (*34, 38*). In the context of germ cell development, proliferation is predominant at day 55, but evidence of meiosis and atresia are also apparent. At day 75, germ cell proliferation is markedly reduced, but both meiosis and atresia are prominent. Additionally, the first signs of follicle formation are evident at day 75 (*38*). Examination of both ages, therefore, would allow investigation of the key processes in germ cell development.

The relationship between gestational nutrition and fetal growth and morbidity has been well established and is well reviewed by Wu and colleagues (*160*). As discussed in Chapter 1, the literature indicates that nutrition restriction early in gestation is less likely to affect overall fetal growth (and morbidity). However, it is important to establish if the nutrition regime used in the current study affects fetal growth and morbidity. Thus, the number and weight of fetuses during, and at completion of the nutrition restriction, is an essential component of this section of work.

Prenatal nutrition has been shown to affect postnatal growth rates (*187, 188*). Importantly, postnatal growth rates can affect the onset of puberty and fertility (*189*). Thus, if differences in growth rates are apparent between the offspring from restricted and maintenance dams, this will have implications for fertility data and require appropriate statistical procedures to model this. Therefore, postnatal growth characteristics are a required element of this work

In regard to female offspring, the onset of puberty was considered a useful variable to determine. A relationship has been established in humans between gestational nutrition and the time of puberty onset (*190*). However, a least one study in sheep reported no effect of gestational nutrition on the timing of puberty onset (*191*). The key indicators of fertility selected to measure were ovulation rate (OR) and AFC. Both variables are proven reliable markers of fertility (*66, 97, 133*). As fertility in pubertal animals differs from mature animals (*84*), these indicators were measured both in pubertal animals and mature animals.

Animals with a low AFC have been shown to have a poor response to superovulation (*63*), similarly in sheep, low AMH levels have been associated with a poor response to superovulation (*192*). Given this, it was hypothesized that ewes from dams exposed to restricted gestational nutrition would display a poor response to a superovulation protocol. To test this hypothesis, a superovulation trial was implemented at the end of the offspring's first breeding season.

2.2 Materials and methods

All animal trials were undertaken in accordance with the 1999 Animal Protection (Codes of Ethical Conduct) Regulations of New Zealand, and with the approval of the Invermay Animal Ethics Committee under the applications 12820, 13098, and 13294. To comply with the regulations and animal ethics approval, animal manipulations were undertaken only by approved users, following standard operating procedures. Internal Drug Administration Orders (IDAO) were prepared and veterinary approval obtained before drugs were administered to animals. To ensure regulatory compliance, regular inspections were undertaken by both representatives of the ethics committee and the Invermay Animal Welfare Officer.

This work was carried out over a 27-month period on: pregnant ewes (referred to as maternal ewes, or dams), day 55 and 75 fetuses, and female offspring up to 19 months of age (referred to as peri-pubertal animals around 8 months of age, and adult ewes around 19 months of age).

Trials were undertaken at the Invermay Research Centre, Mosgiel, New Zealand (45.9^{o}S, 170.3^{o}E).

2.2.1 Pre-treatment, selection and mating of animals

Five-year-old Romney x Texel ewes (n =150) were initially selected for this study. The absence of known genotypes affecting fertility was an important consideration in animal selection as this has the potential to influence subsequent results. Additionally, animals with a history of producing single and twin lambs, but not triplets, were selected. It was considered that the metabolic stress of supporting triplet fetuses during a nutrition restriction period, even during the first trimester, may have a negative effect on animal welfare. An overview of all trials is presented in Figure 2.1.

Over a three-month period, the pasture management of these ewes was intensively monitored with the aim of standardising the ewe's body condition scores (BCS) and body weights (target BCS 3.0 to 3.5, target weight 65 to 70 kg). At the onset of the breeding season (March, 2013), 50 ewes whose weights were furthest, either side of the mean weight, were discarded from the study.

The oestrous cycles of the remaining 100 ewes were synchronised. Synchronisation is achieved through the administration of exogenous progesterone. Exogenous progesterone was introduced using intrauterine progesterone releasing devices (0.3 g progesterone, Eazi-Breed CIDR Sheep and Goat Insert, Zoetis NZ, Auckland, NZ). Endogenous progesterone is produced by the corpora lutea (CL) following ovulation. Continued production of progesterone following successful mating (resulting in early embryo development) then maintains pregnancy. The resulting extended period of elevated progesterone mimics early pregnancy and prevents ovulation. Sheep display estrus approximately 36 hours following CIDR removal (*193*).

2013	February-April: Pre-selection and treatment of dams March-April: Synchronisation April: Mating to intact rams April-June: Feed restriction June: Collection of day 55 fetuses July: Collection of day 75 fetuses September: Lambing
2014	March-July: Monitoring offspring for onset of puberty April-May: Laparoscopy and ultrasound scanning July: Superovulation
2015	February-June: Monitoring offspring for onset of estrus March-April: Synchronisation April: Daily blood sampling throughout oestrous cycle April: Intensive blood sampling (every 15 minutes for 8 hours) April-May: Laparoscopy and ultrasound scanning May: Euthanasia of remaining animals

Figure 2.1 Summary of animal trials.

Figure 2.2 gives an overview of the synchronisation method used for the dams. In this instance, CIDRs were inserted for 10 days, after which they were replaced with new CIDRs for a further 3 days. Mating to intact rams to produce pregnancy was undertaken during the second cycle following synchronisation. This protocol produces a comparatively loose synchronisation enabling subsequent fetal collections to be spread over a 2 to 3-day period. For female offspring at 19 months of age, a tight synchronisation was preferred to allow experiments to be performed at specific stages of the cycle. To achieve this, at CIDR withdrawal, prostaglandin was administered (0.7 mL subcutaneous of Estrumate, MSD Animal Health, Upper Hutt, NZ). Prostaglandin causes regression of any CLs present, consequently the removal of both exogenous (CIDRs) and endogenous (CLs) progesterone simultaneously results in a tight synchronisation.

Following CIDR removal, vasectomised rams fitted with a mating harness and crayon were introduced to the flock, and mating marks were recorded each morning. Day 1 of an animal's oestrous cycle was defined as the day on which a mating mark was first recorded, as mating marks result from mating activity in the previous 24 hours. A further 20 animals that either did not respond to, or had a delayed response to the synchronisation protocol, were removed from the trial.

Four days following the introduction of vasectomised rams, the flock was introduced to the indoor diet. This was incrementally introduced to the 80 remaining ewes over an 8-day period. Access to pasture was decreased over 4 successive 2-day periods as the indoor diet was increased from 25% to 50% to 75%, and finally 100% of the dietary requirement. A single ewe failed to adjust to the indoor diet and, following veterinary advice, was removed from the trial. The remaining 79 ewes were ranked by their weights. Odd rankings were assigned to the restricted feed group (n = 40), and even rankings were assigned to the maintenance group (n = 39). Animals were moved indoors and all were fed a maintenance diet for 5 days.

At the completion of the acclimatisation period, ewes were exposed to intact Texel rams fitted with a harness and marking crayon. This exposure occurred during the day. At the end of each day, rams were removed and the mating marks recorded. All ewes were mated over a 4-day period. In this instance, as the mating marks were the result of mating over the previous 6 to 8 hours, the day of mating was designated as day 0. From day 0, those animals assigned to the restricted feed group (n = 40) were placed on a diet equivalent to 0.6 of maintenance, the remaining animals (n = 39) continued their maintenance diets. Assigned diets were maintained for 55 days from the day of mating.

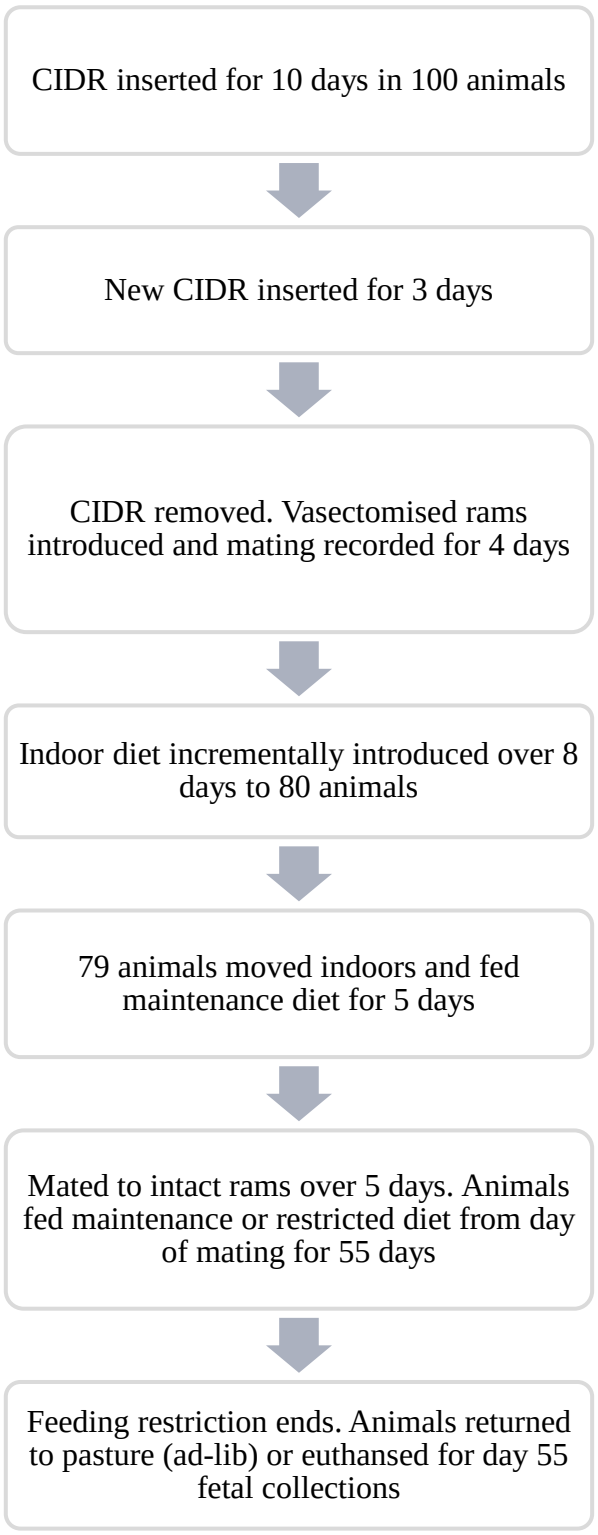

Figure 2.2 Workflow for maternal ewes from synchronisation until day 55 of gestation.

2.2.2 Body weights and body condition scores

Throughout this trial, both live weight and BCS were recorded. Both measurements are good indicators of an animal's health, and can effectively show changes in an animal's condition in response to a number of factors, including nutrition. While live weight shows an animal's overall response, BCS assesses the level of fat over the skeletal muscle, indicating the animal's energy reserve.

Weights were measured using scales mounted permanently in a pneumatic crush, and recorded on an electronic head set (Tru-test XR3000, Tru-test group, Auckland, NZ). The system has an accuracy of ± 1%. To exclude any potential negative effects of the weighing procedure on fetuses, pregnant ewes beyond 90 days of gestation were not weighed.

BCS is assessed by light palpation of the ewe in the lumbar region, on and around the backbone, in the loin area immediately behind the last rib, and above the kidneys. Body condition scoring involves assessing the fat and muscle cover of an animal by the prominence (sharpness) of a number of skeletal features associated with the spine. Body condition scores range from 1 to 5. A score of 1 (where it is not possible to detect any muscular or fatty tissue between the skin and the bone), indicates an animal is extremely emaciated and on the point of death. A score of 5 (where the spine cannot be felt) indicates a severely obese animal (*194*). To minimise variation, BCS was recorded by two personnel with extensive experience in this technique.

2.2.3 Blood sampling

For both maternal ewes and female offspring, blood samples were collected by jugular vein puncture. Vacutainer tubes containing lithium heparin anti-coagulant (Becton Dickinson, Franklin Lakes, NJ, USA) were used to collect blood samples. Samples were kept cool and within two hours of collection, the blood was centrifuged at 1300 g for 15 minutes (Eppendorf 5810R, Medi'Ray Ltd, Auckland, NZ). The plasma was recovered and frozen at -20°C until required.

To determine the patterns of gonadotrophin secretion in adult female offspring, the collection of serial blood samples was required. For this study, samples were collected every 15 minutes over an 8-hour period from a subset of 10 maintenance and 10 restricted female offspring. Each animal was fitted with an in-dwelling jugular catheter. Following a 2 mL subcutaneous (sc) injection of local anaesthetic (Lopaine, 2% lignocaine hydrochloride, Ethical Agents, Auckland, NZ), a sterile stainless steel 12 gauge needle was inserted into the jugular vein. Vinyl tubing (SV70, internal diameter 1 mm, external diameter 1.5 mm, Dual Plastics, Auburn, NSW,

Australia) was threaded through the needle into the jugular vein. Following removal of the 12 gauge needle, the tubing was attached to a holding device sutured to the skin of the neck overlying the jugular. The end of the tubing was bandaged to an accessible area at the base of neck, between the shoulders. Serial blood samples were drawn from the tubing using a 5 mL syringe. Following the addition of 0.1 mL of sodium heparin (50 IU/mL) samples were processed as described above.

2.2.4 Diet calculations

Diets were based on an animal's daily metabolisable energy (ME) requirements, which in turn is related primarily to the animal's weight. Based on advice from the AgResearch Animal Facilities Unit, Grasslands, Palmerston North, the formula used to calculate an animal's ME requirement was

$$0.6 \times \text{ewe weight (kg)}^{0.75}$$

This formula is in line with other dietary calculations for sheep summarised by Wallach (*195*) and Dryden (*196*). For the ewes in the current study (average weight of 70 kg), the formula estimates the daily ME requirements of each ewe at 14.5 megajoules of metabolisable energy (MJME).

In order to provide both the animal's daily energy requirements and sufficient fibre to allow for healthy rumen function, the diet consisted of a mixture of three commercial products in the following proportions. 50% was a silage product (Fibre Eazy, Fibre Fresh Feeds Ltd, Reporoa, NZ), 25% was a pellet product (Sheep Pellets, Reliance Stockfoods, Dunedin, NZ) and the remaining 25% was lucerne chaff (Sergeant Dan Stockfoods, Gore, NZ).

Different feeds have different metabolisable energy (ME) content, expressed as mega joules (MJ) of metabolisable energy per kg of dry matter. For example, pasture would normally range between 8 to 12 MJ ME per kg of dry matter. This value is dependent on the type and quality of pasture. Commercial feeds have a more stable ME content. ME values and dry matter content for each product were provided by the manufacturer (Table 2.1).

Table 2.1 illustrates the calculation used to determine the quantity of each feed required to meet the daily energy requirements, based on a diet consisting solely of each product. Table 2.2 shows the quantity of each feed required in the mixed diet for both maintenance and restricted (0.6 of maintenance) ewes.

Table 2.1 Daily feed requirements

Product	ME value	Dry matter content	Calculation	Daily requirement (kg) for a 70 kg ewe
Fibre Eazy	8.5	50%	(14.5/8.5) x (1/0.5)	3.4
Sheep Pellets	9.7	90%	(14.5/9.7) x (1/0.9)	1.7
Lucerne Chaff	8.0	85%	(14.5/8.0) x (1/0.85)	2.1

Daily feed requirements for each type of feed for a 70 kg ewe who's daily ME requirement is 14.5 MJME. This calculation is based on a diet comprising solely of each product.

Table 2.2 Diet composition from day 0 to day 55 of gestation

Product	% of total diet	Maintenance requirement (kg)	Restricted requirement (0.6 maintenance) (kg)
Fibre Ezy	50%	1.7	0.85
Sheep Pellets	25%	0.42	0.25
Lucerne Chaff	25%	0.53	0.32

The values represent the daily allocation for each ewe from the maintenance and restricted groups.

2.2.5 Housing and feeding

Ewes were housed in pens containing between 5 and 9 animals of the same group. A minimum of 2.5 m^2 was allocated per sheep in accordance with the New Zealand Ministry for Primary Industries guidelines. Ad libitum access to water was provided for each pen. Ewes were fed from bins, with one bin allocated for each ewe. Close monitoring was undertaken during feeding to ensure that all ewes received their designated feed allocation. Ewes were fed twice daily (morning and afternoon) with pens being cleaned following each feeding. At the completion of 55 days of restricted or maintenance nutrition, animals were returned to pasture and ad libitum feeding.

2.2.6 Ultrasound scanning

Trans-rectal ultrasound scanning was performed on pregnant ewes at day 35 to determine fetal status. Additionally, the procedure was performed on female offspring to determine both ovulation rate (OR), and the number of antral follicles > 2 mm present (AFC). The procedure has previously been used to measure both OR and AFC in sheep (*197*). In accordance with animal ethics requirements, the procedure followed the AgResearch standard operating procedure (INV036) and was performed by an approved operator. Briefly, animals were restrained in a dorsally recumbent position. Following introduction of approximately 50 mL of lubricant into the rectum, the transducer was inserted. Ultrasound images indicating pregnancy status was determined by manipulation of the transducer. Trans-rectal ultrasound scanning was performed with an Aloka SSD 900 scanner with a 7.5 MHz trans-rectal probe (Hitachi-Aloka Medical Ltd, Japan).

2.2.7 Laparoscopy

Laparoscopy is a well proven and efficient method of accurately determining ovulation rates (*198, 199*). In accordance with animal ethics requirements, the procedure followed the AgResearch standard operating procedure (INV003) and was performed by an approved operator. Briefly, food and water were withheld overnight prior to surgery. Animals were sedated with 1.0 mL (sc) of Acezine10 (1.4% acepromazine maleate, Ethical Agents, Auckland, NZ). Animals were restrained in a dorsally recumbent position. The abdominal area was shaved and washed with 70% ethanol. Incision sites were injected sc with 2.0 mL of lopaine local anaesthetic (2% lignocaine hydrochloride. Ethical Agents, Auckland, NZ). A trochar and cannula were used to penetrate the left side of the abdominal wall and the abdomen inflated with CO_2. The laparoscope was introduced through the cannula and manipulating forceps

introduced through a small scalpel incision made through the right side abdominal wall. Following visualisation of the ovary, the incision sites were treated with an iodine solution (Vetadine Iodine Animal Wash, Bayer Animal Health, NZ) and animals were returned to pasture.

2.2.8 Euthanasia and fetal sampling

Pregnant ewes were euthansed at day 55 (n = 24) and day 75 (n = 18) of gestation until at least 6 female fetuses with a unique dam were recovered from each nutritional group for each age. Euthanasia of all animals was by intra-venous barbiturate overdose (Pentobarb 500, Provet NZ Pty Ltd, Auckland, NZ) at a dose rate of 0.3 mL per kg of body weight. In the case of pregnant animals, the barbiturate was administered slowly (approximately 30 seconds) allowing the drug sufficient time to cross the placenta and euthanase the fetuses. Upon expiration of pregnant ewes, fetuses were removed. Blood was collected from the day 75 fetuses by cardiac puncture using a 20 gauge needle and 5 mL syringe. Sodium heparin (0.1 mL of a 50 IU/mL solution) was immediately added to each blood sample. Within 30 minutes, the samples were centrifuged and stored as described in Section 2.2.3. Fetal crown-rump lengths and weights were recorded. Fetal tissue samples (gonads, kidney and mammary gland) were collected and weighed within 10 minutes of euthanasia. The ovaries were either fixed in 4% paraformaldehyde at 4°C for 24 hours (left ovary) and then processed for histology, or snap frozen in liquid nitrogen before storage at -80°C until required (right ovary).

2.2.9 Lambing

Thirty seven animals progressed through to produce 34 female lambs (13 from dams exposed to restricted nutrition and 21 from maintenance dams). Assigning lambs to the correct mother was a critical aspect of this study. Throughout the lambing period, ewes were monitored twice daily by an experienced shepherd. The number, sex, and weight of lambs was recorded. Lambs were assigned to mothers and tagged for ongoing identification.

2.2.10 Onset of puberty in female offspring

Prior to the onset of the first breeding season (March, 2014), vasectomised rams fitted with a mating harness containing a marking crayon were introduced to the ewes. Ewes were checked twice weekly for mating marks to indicate the onset of puberty. Twice weekly checks continued throughout the duration of the breeding season to determine the onset of anestrus.

2.2.11 Superovulation of female offspring

The superovulation trial of female offspring was undertaken towards the end of the first breeding season (July, 2014) so as the administration of drugs would not to affect the natural cyclicity of animals during the onset of puberty. The superovulation protocol used in this study is a standard procedure (*200*), and is outlined in Figure 2.3. The procedure involved synchronisation of animals as described in Section 2.2.1. At CIDR change, animals received an injection of 400 IU Pregnant Mares Serum Gonadotrophin (Folligon, MSD Animal Health, Upper Hutt, NZ) followed by eight twice daily injections of 20 mg Porcine Follicle Stimulating Hormone (Folltropin V, Bioniche Animal Health) over four days. As the ewes were no longer cycling naturally, Pregnant Mares Serum Gonadotrophin (PMSG) was used to help stimulate ovarian activity. PMSG also contains higher LH concentrations than commercial FSH products, and is often used in conjunction with FSH products to ensure ovulation (*193, 200*). Follicle Stimulating Hormone (FSH) stimulates the growth of more follicles resulting in higher ovulation rates. Typically a 4 to 5 fold increase in OR is observed in sheep, although results can be highly variable between animals (*201*).

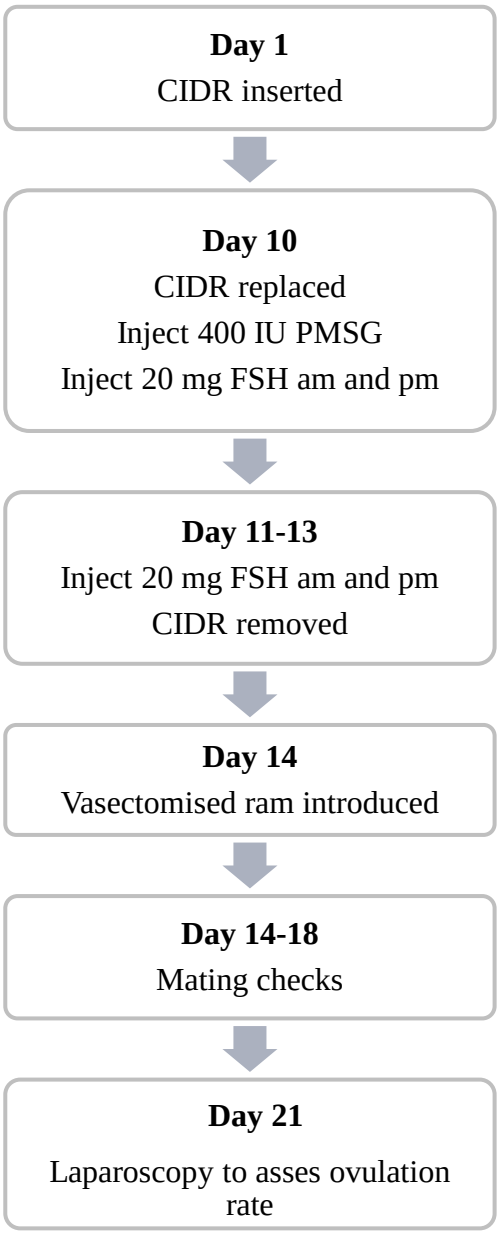

Figure 2.3 Superovulation protocol for female offspring.

2.2.12 Statistics

With the exception of gene expression data, the same statistical principles and tests were applied to the data in each chapter. Statistical approaches to gene expression data are described in Chapters 5 and 6. Statistical procedures were performed using the Genstat statistical software package (17th edition, VSN International, Hemel Hempstead, UK).

Where a continuous time variable was present, a repeated measures analysis of covariance (RMANCOVA) was applied (e.g. gestational and postnatal weights). On occasions, additional variables were also included in the model (e.g. progesterone concentrations throughout the oestrous cycle where OR was also included in the model). RMANCOVA also tests for an interaction between the variables within the model. A significant age x nutritional group interaction indicates age is affecting the variable being analysed differently between the two groups. Figure 2.4 illustrates hypothetical weight and age data for two groups. Graph A would return no significant interaction, while graph B would return a significant interaction.

Where a discrete time/age variable was present, a general linear model (GLM) was applied with age/time and nutritional group in the model. GLM is a robust test suitable for a wide range of data, including unbalanced data and where multiple variables require consideration. Where GLM indicated a significant interaction or a trend ($p < 0.1$), then analysis of variance was applied to analyse either groups or ages separately. Multiple range tests are reported sparingly as these increase the probability of type 1 errors (i.e. false positives). The Bonferroni multiple range test is reported only where differences followed a clear pattern related to the biology.

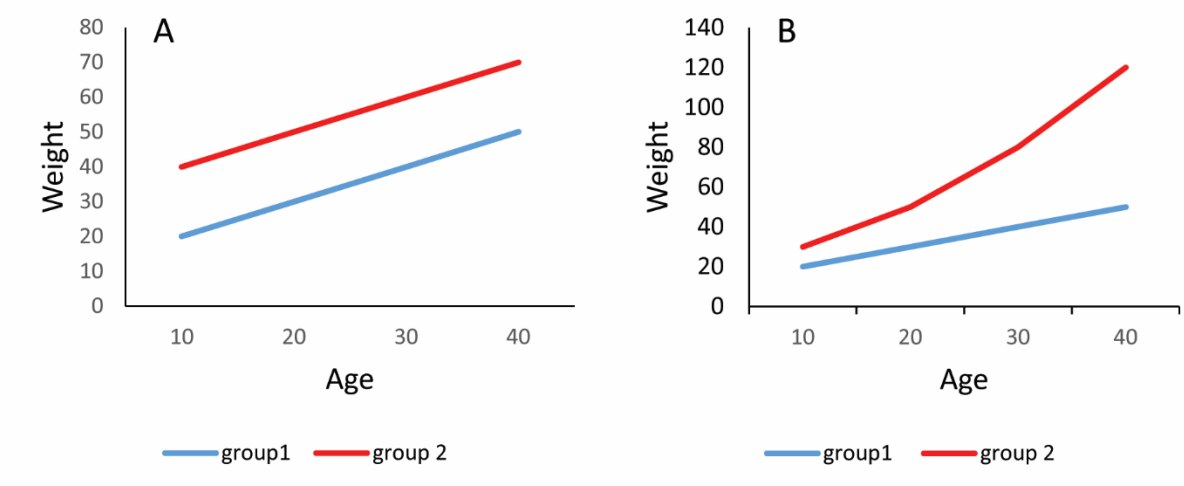

Figure 2.4 Hypothetical data illustrating the concept of interactions between variables. **(A)** As age affects weight in both groups in a similar fashion, no interaction would be apparent. **(B)** As age increases, a more pronounced effect on weight is apparent in group 2, and a significant age x weight interaction is evident.

2.3 Results

2.3.1 Maternal weights during gestation

All 79 animals assigned to the gestational nutrition trial mated over a 5-day period, with restricted and maintenance nutrition regimes started from the day of mating. At the time feeding regimes were introduced, the average weight of the 39 animals assigned to the maintenance group was 70.3 ± 0.6 kg (maximum 73 kg, minimum 63 kg) with an average BCS of 3.2. The average weight of the 40 animals assigned to the restricted group was 70.4 kg ± 0.6 kg (maximum 79 kg, minimum 63 kg) with an average BCS of 3.3. The BCS scores of 3.2 (maintenance) and 3.3 (restricted) at the beginning of the nutrition regimes indicates that these animals were in good condition, showing no signs of either obesity or malnutrition. At cessation of the nutrition regimes and return to ad libitum feeding (day 55 of gestation), maintenance animals weighed 72.4 kg ± 0.6 (gain of 2.1 kg), and their BCS remained at 3.2. By comparison, the restricted animals on average had lost 5.0 kg, completing the restricted nutrition regime with an average weight of 65.4 kg ± 0.5 and a BCS of 2.6 (a loss of 0.7 in BCS). Maternal weights during early to mid-gestation are presented in Figure 2.5.

RMANCOVA analysis indicated significant effects of stage of gestation ($p < 0.01$), and group ($p < 0.01$) on body weight, with a significant group x age interaction ($p < 0.05$). The interaction reflects the marked decline in weights of the restricted animals at the beginning of the restriction period and also the post restriction increased growth plane evident in the restricted animals. The drop in BCS in restricted animals from 3.3 to 2.6 between day 55 and 75 was significant ($p < 0.05$, ANOVA), suggesting increased use of energy reserves in the restricted animals in response to the nutrition restriction.

From day 55, at the completion of restricted nutrition, through until the final recorded gestational weight at day 90, restricted animals showed a significantly steeper growth plane than maintenance animals. During this period, restricted animals gained an average of 5.9 ± 0.6 kg, while maintenance animals gained an average of 4.3 ± 0.7 kg ($p < 0.05$, ANOVA).

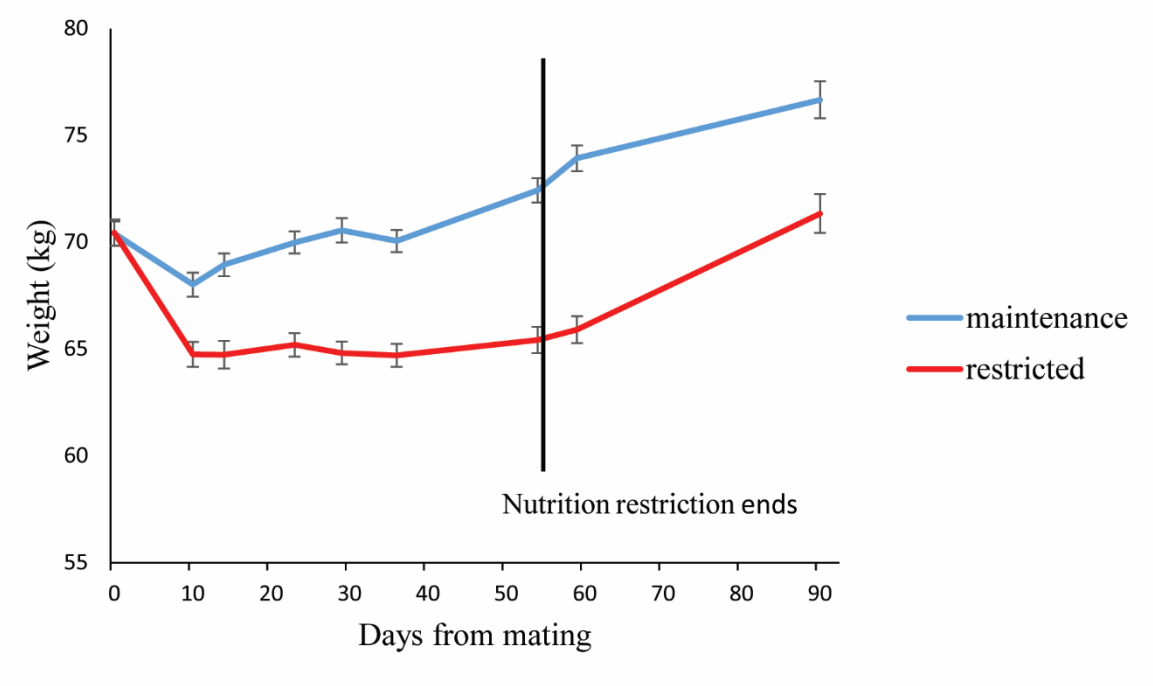

Figure 2.5 Maternal weights during early to mid-gestation. Nutrition restriction began at day 0 and finished at day 55.

2.3.2 Fetal weights

The number of fetuses detected by ultrasound scanning at day 35, and the number of fetuses recovered at days 55 and 75 are presented in Table 2.3. There was no effect of group or gestational age on the number of fetuses present.

Table 2.3 Fetal numbers present during gestation

Gestational age	Maintenance	Restricted
Day 35	1.6 ± 0.1 n = 39	1.5 ± 0.1 n = 40
Day 55	1.5 ± 0.2 n = 7	1.6 ± 0.1 n = 8
Day 75	1.9 ± 0.3 n = 7	1.5 ± 0.2 n = 8

n = number of ewes. Fetuses at day 35 were estimated by ultrasound scanning. Fetuses at days 55 and 75 were recovered from a subset of animals following euthanasia.

Size and weight data for day 55 fetuses is shown in Table 2.4, and for day 75 fetuses in Table 2.5. There was no effect of group on the crown rump length, fetal weight, or the weights of the organs recovered at either gestational age. This is consistent with the concept that the nutritional regime had no effect on overall fetal growth at day 55 and 75 of gestation. This also indicates that the post restriction compensatory growth shown by those ewes exposed to restricted nutrition did not result in increased fetal growth.

Additionally, there was no effect of fetal sex on the size, or weight of the fetuses, although a significant sex effect was observed on gonad weight, fetal testes being significantly heavier then fetal ovaries ($p < 0.01$, ANOVA). This result is consistent with two previous studies in sheep sharing the same cohort of animals (*38, 202*).

Table 2.4 Day 55 fetal and organ weights

Female	**Maintenance (n = 7)**	**Restricted (n = 8)**
Crown - rump length (cm)	10.4 ± 0.1	10.2 ± 0.1
Fetal weight (g)	37.9 ± 0.9	36.5 ± 1.0
Average gonad (g)	0.0090 ± 0.001	0.0092 ± 0.001
Kidney (g)	0.24 ± 0.01	0.20 ± 0.02
Mammary (g)	0.058 ± 0.008	0.046 ± 0.001
Male	**Maintenance (n = 8)**	**Restricted (n = 11)**
Crown - rump length (cm)	10.2 ± 0.1	10.4 ± 0.1
Fetal weight (g)	37.4 ± 1.3	38.4 ±1.3
Average gonad (g)	0.016 ± 0.001	0.023 ± 0.007
Kidney (g)	0.212 ± 0.018	0.232 ±0.011

Values are means and standard errors.

Table 2.5 Day 75 fetal and organ weights

Female	**Maintenance (n = 7)**	**Restricted (n = 8)**
Crown - rump length (cm)	18.7 ± 0.2	18.6 ± 0.3
Fetal weight (g)	232.9 ± 4.8	227.5 ± 8.6
Average gonad (g)	0.024 ± 0.002	0.026 ± 0.001
Kidney (g)	1.39 ± 0.05	1.50 ± 0.09
Mammary (g)	0.65 ± 0.07	0.56 ± 0.09
Male	**Maintenance (n = 6)**	**Restricted (n = 4)**
Crown -rump length (cm)	19.0 ± 0.3	18.2 ± 0.5
Fetal weight (g)	246.4 ± 11.2	219.1 ± 10.6
Average gonad (g)	0.062 ± 0.005	0.055 ± 0.001
Kidney (g)	1.42 ± 0.14	1.40 ± 0.010

Values are means and standard errors.

2.3.3 Lambing and postnatal growth

Following fetal collections, 35 ewes (21 maintenance and 14 restricted) progressed through to lambing. No group differences were observed at lambing in either the number of lambs born per ewe, weights of the lambs, or the ratio of female:male lambs (Table 2.6).

Of the 34 female lambs born, 4 did not survive through to puberty. Growth of the 30 remaining female offspring from birth to 560 days of age is shown in Figure 2.6. There was a significant age effect ($p < 0.01$, RMANCOVA), but no group effect on weight, and no significant group x age interaction, indicating that the restricted nutrition did not have an effect on postnatal growth of the female offspring.

Combined, the results from Sections 2.3.2 and 2.3.3 show that the nutrition restriction had no effect on fetal growth, fetal survival or postnatal growth of female offspring.

Table 2.6 Number of lambs born and lamb weights

Group	Variable	Lambs born	Female	Male	Lambs per ewe
Maintenance	Number	39	21	18	1.86
	Weight (kg)		5.5 ± 0.3	5.6 ± 0.3	
Restricted	Number	26	13	13	1.86
	Weight (kg)		5.4 ± 0.3	5.8 ± 0.2	

Data based on 21 maintenance and 14 restricted ewes.

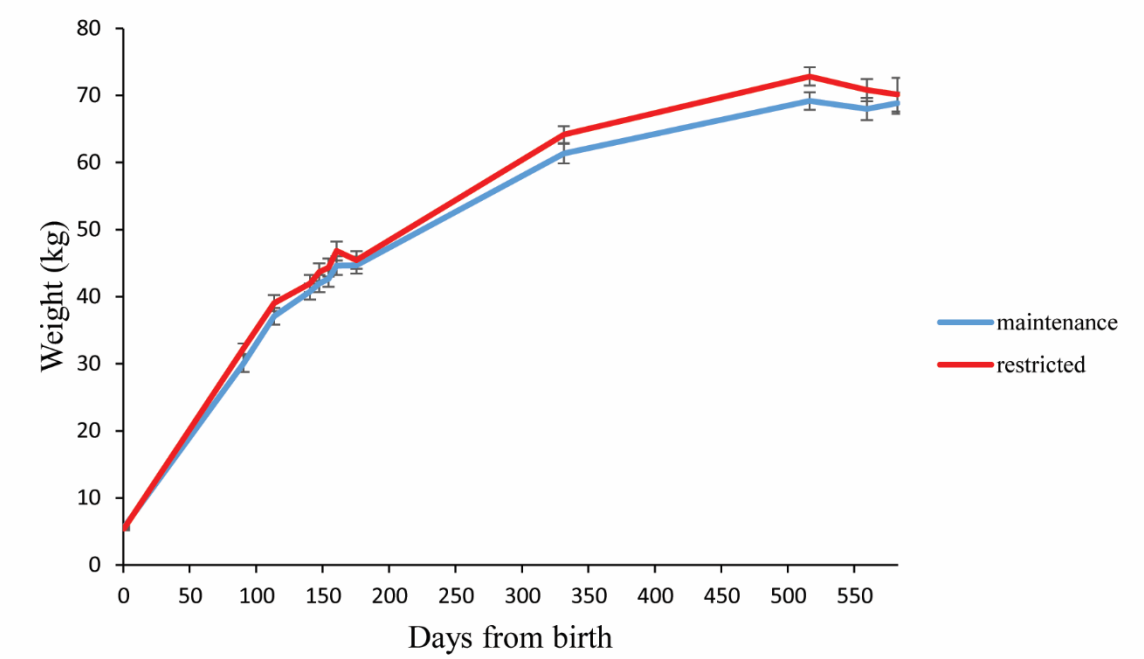

Figure 2.6 Postnatal weights of female offspring. Values are means and standard errors for 17 maintenance and 13 restricted animals.

2.3.4 Onset of puberty

Of the 30 remaining female offspring (17 maintenance and 13 restricted), 3 maintenance animals failed to display estrus, i.e. no mating marks were recorded over the 3-month period (March to May, 2014). Of those animals which displayed estrus, there was no difference in the time from birth until the first recorded mating mark ($p = 0.2$, ANOVA). For maintenance animals, this was an average of 218 ± 5 days (minimum 185, maximum 241), while for the restricted animals, this was an average of 225 ± 3 days (minimum 211, maximum 240).

2.3.5 Superovulation of female offspring

A relationship between AFC and superovulation response has been demonstrated previously in cattle, with low AFC being associated with a poor superovulatory response (*203*). Similarly in sheep, a correlation between plasma AMH concentrations and superovulation response has been demonstrated (*192*). Given the association between restricted gestational nutrition and postnatal AFC, the response to superovulation in offspring from restricted and maintenance dams was examined. Following administration of the superovulation protocol, no animals displayed estrus. Laparoscopy of 10 randomly selected animals 6 days following the final FSH

injection indicated that all 10 animals had failed to ovulate. It is unclear whether this failure was due to issues related to the age of the animals (being relatively young), the timing of the experiment (as most animals had moved into anestrus), or a product failure. Laparoscopy of 10 randomly selected animals showed a high level of follicular activity suggesting that increased FSH levels had been achieved. The implication is that the failure is related to PMSG, with either an inappropriate dose being administered for these animals or an issue related to product quality.

2.3.6 Indicators of fertility: OR and AFC

Indicators of fertility were assessed by laparoscopy (OR) and ultrasound scanning (OR and AFC) at 8 months of age (pubertal) and 19 months of age (adult). A synchronisation protocol was not utilised in the offspring at puberty to ensure a natural onset of puberty. Adult offspring were synchronised as described in Section 2.2.1. Measurements were undertaken over 4 consecutive cycles (laparoscopy on cycles 1 and 3, ultrasound scanning on cycles 2 and 4). The day of cycle varied between pubertal animals but was restricted to day 5 or 6 for adult offspring to coincide with the emergence of the first follicle wave.

OR and AFC data is shown in Table 2.7 for peri-pubertal offspring, and Table 2.8 for offspring at 19 months of age. Data presented is the combined data for two observations for each technique used (laparoscopy and ultrasound scanning). Including data from both ages and groups there was a significant age effect and group effect on both OR and AFC ($p < 0.05$ for all comparisons, GLM). There was no significant age x group interaction ($p = 0.07$ for OR, and $p = 0.075$ for AFC, GLM). While a significant group x age interaction was not recorded the presence of a trend ($p < 0.1$ for both OR and AFC) justifies the use of ANOVA to analyse each age separately.

No significant group differences in AFC or OR were noted in peri-pubertal animals. However, in contrast to predicted results, at 19 months of age, ewes from dams exposed to restricted nutrition had significantly higher OR and higher AFC when compared to those ewes from maintenance dams (Table 2.8). Ewes from restricted dams showed a 26% increase in OR (regardless of the technique used) and a 44% increase in AFC.

Additionally, irrespective of group, peri-pubertal animals had a significantly higher AFC when compared to animals at 19 months of age ($p < 0.01$, ANOVA). In contrast, while the OR rate in peri-pubertal animals appeared lower than in adult animals, this did not achieve significance with the number of animals in this study ($p = 0.07$).

Table 2.7 OR and AFC at 8 months of age

Method	Fertility indicator	Maintenance N = 17	Restricted N = 13	P value
Laparoscopy	OR	1.2 ± 0.1	1.5 ± 0.2	0.6
Ultrasound scanning	OR	1.5 ± 0.1	1.4 ± 0.2	0.4
Ultrasound scanning	AFC	17.3 ± 1.3	19.6 ± 2.6	0.8

Data are means and standard errors for two combined replicates for each technique. P values derived from ANOVA for between group comparisons.

Table 2.8 OR and AFC at 19 months of age

Method	Fertility indicator	Maintenance N = 17	Restricted N = 13	P value
Laparoscopy	OR	1.6 ± 0.1	2.1 ± 0.1	< 0.01
Ultrasound scanning	OR	1.6 ± 0.1	2.1 ± 0.2	0.02
Ultrasound scanning	AFC	6.8 ± 0.5	9.8 ± 0.6	< 0.01

Data are means and standard errors for two combined replicates for each technique. P values derived from ANOVA for between group comparisons.

2.4 Discussion

The primary objective of this work was to establish a model where restricted gestational nutrition affected postnatal fertility. This objective has been met, although, the results are contrary to expectations and to most previous studies.

An increase in AFC and OR at 19 months of age in animals from dams exposed to restricted gestational nutrition is consistent with fertility being increased in these animals. A major limitation of this study is that both OR and AFC represent indicators of fertility and not fertility per se. While OR is considered a strong indicator of fertility (*84*), and an association between AFC and fertility has been demonstrated (*63*), direct measurement of fertility requires the female offspring to be mated to intact rams and the number of lambs born from these matings recorded. While this experiment was considered, it was deemed impracticable, particularly given the limited number of animals available.

The results pose two major questions worthy of discussion. Firstly, why are increased indicators of fertility apparent in adult ewes from restricted dams where previous studies report decreased fertility? Arguably, the three variables likely to account for the unexpected results in this study are: a) the degree of nutrition restriction, b) the timing of nutrition restriction, and c) the post-restriction diet.

Most published studies in sheep use a restriction equivalent to 50% of maintenance (*170, 178, 184*), whereas in the current study, dietary restriction was calculated at 60% of maintenance. While the difference between 60% and 50% of maintenance may appear small, effects on the animal can be profound. In this study, pregnant ewes exposed to restricted nutrition lost 7% of their body weight over the 55-day period. Studies using 50% of maintenance over a similar time period resulted in a loss of 16% of body weight (*178*). Potentially, the less severe restriction used in this study may not have been sufficient to induce changes that result in negative effects on postnatal fertility.

The timing of nutrition restriction also varies considerably between studies. In this study, nutrition restriction ended at day 55 of gestation, just as key germ cell processes such as meiosis and apoptosis are becoming prevalent (*38*). The selection of this time period (day 0 to day 55) may therefore have avoided any prospective negative impacts of restricted nutrition on these key processes.

Following the restricted nutrition period, animals in this study were fed ad libitum. The majority of published studies either return animals to a maintenance diet (*178, 204, 205*) or fail to specify

the post-restriction diet. The ad libitum diet used from day 55 in this study is likely to have allowed the restricted animals to go through a compensatory catch up phase as shown in Figure 2.5. This compensatory phase is occurring over the critical time period where germ cells undergo meiosis, apoptosis is prevalent, and the first follicles are forming. The potential for a "growth spurt" from day 55 to have a positive effect on these key processes seems plausible.

The second major question posed by these results is why are these positive effects on indicators of fertility apparent in adult animals and not in peri-pubertal animals? It is a recognised issue in the New Zealand sheep industry that mating of sheep at puberty produces poor results when compared to adults (*206*). Studies have shown that poor lambing from these animals is a result of lower ovulation rates and lower embryo survival (*84*), suggesting a difference in the ovaries between adults and peri-pubertal animals. These differences may be inherent ovarian differences, or driven by differences in gonadotrophin secretion. The concept of differences between the ovaries of pubertal animals compared to adult animals is supported by the ultrasound scanning results which highlight a higher AFC in peri-pubertal animals compared to adult animals. Bartlewski and colleagues reported a rise in AFC just prior to puberty in sheep (*207*), with this rise being attributed to an increase in LH pulse frequency, most likely a response to exposure to rams. This is unlikely to be the case in pubertal animals used in this study, as initial exposure to rams occurred more than 60 days prior to assessment of AFC.

While differences in the pubertal ovary compared to the adult ovary may offer some insights into why effects are seen in adult but not pubertal animals, the finding of high AFC in pubertal animals where fertility is low, seems counter to the studies linking high AFC with high fertility. One possible explanation accounting for both the high AFC-low fertility issue in peri-pubertal animals, and the lack of nutrition induced differences observed in pubertal ovaries, is the proposed dual recruitment of granulosa cell theory demonstrated in rodents (*40*). This theory proposes that during fetal development, granulosa cells are recruited in two waves. The first wave corresponds to initial contact between germ cells and somatic cells, equivalent to days 30 to 50 in the sheep. A subsequent wave of recruitment of granulosa cells occurs later, following formation of germ cell nests or cords. This is equivalent to days 50 to 75 in sheep. The theory contends that follicles growing before puberty contain granulosa cells primarily from the first wave of recruitment. A gradual shift in follicular granulosa cell make-up occurs such that, postpuberty, growing follicles contain granulosa cells primarily from the second wave of recruitment. A supporting study also illustrates that prepuberty and postpuberty follicle dynamics are different, with prepuberty follicles growing significantly faster than postpuberty

follicles (*85*). Should such a theory hold in sheep, then the differences in prepubertal follicle dynamics may simply negate or mask the differences observed in adults. The mechanism by which AFC is increased in adults from restricted dams may not produce an additive effect to the mechanism driving higher AFC in pubertal ovaries. Alternatively, only the postpubertal growing follicles may be affected by the changes to gestational nutrition, which by extension, suggests prenatal nutritional effects are limited to follicles from the second wave of recruitment. As this wave of recruitment largely occurs in the post-restriction period, this would support the concept that it is this period of diet change, and not the restriction by itself that may be important in establishing the effects observed.

The gestational nutrition regime used in this study did not have any effect on the growth or survival of fetuses, nor on the growth of female offspring. While numerous studies across a range of species show differing results, this finding is in agreement with previous studies in sheep which use nutritional restriction during early to mid-gestation (*178, 204, 205*). Effects of restricted gestational nutrition on fetal size and postnatal growth are only evident when extended periods of restricted nutrition are employed that encompass the later stages of gestation (*208, 209*).

In summary, the restricted gestational nutrition regime used in this study (0.6 of maintenance from day 0 to day 55 of gestation), increased indicators of fertility in adult female offspring at 19 months of age. As these results are contrary to previous studies, supporting evidence is critical to reinforce these findings. Characterisation of hormone profiles in maternal, fetal and especially in the female offspring, may provide evidence to support these novel findings and potentially offer insights into the underlying mechanism(s) responsible for the observed effects.

Chapter 3 . Measurement of Hormones and Metabolic Factors

3.1 Introduction

Hormones and metabolic factors have been related to an array of functions and conditions including menopause (*210*), ovarian function (*131*), ovarian insufficiency (*211*), appetite (*212*), and nutrition (*213*). Thus, the potential of hormone and metabolic profiles to contribute to the results observed in this study to date, and to aid our understanding of possible mechanisms is without question.

Hormone concentrations in both fetal and maternal plasma, as well as their metabolic status, can illustrate effects of restricted nutrition on both fetal and maternal animals. Further, they may provide insights into the effects on the developing fetus, and the fetal ovary which may contribute to the observed outcomes in female offspring.

In maternal plasma, leptin appears a clear choice to measure in these samples. Leptin is a hormone whose primary function is regulation of energy homeostasis by inhibiting hunger (*214*). However, it also has a number of roles in the reproductive system (*215*). In general, leptin stimulates at the level of the hypothalamus (*152, 216*), increasing the pulsatile secretion of GnRH. In the ovary, elevated leptin levels are thought to increase ovulation rate in sheep (*155, 217*). Studies have demonstrated naturally elevated maternal leptin levels during pregnancy in sheep and primates (*218, 219*). Additionally, increased nutrition during gestation is associated with elevated leptin levels (*220*), while restricted gestational nutrition decreases expression of leptin mRNA (*205*) in adipose tissue, the major source of circulating leptin.

Steroids, in particular progesterone, are also important elements found in maternal plasma, with progesterone being primarily responsible for the maintenance of pregnancy (*221*). Decreased plasma concentrations of progesterone have been reported in overfed ewes (*222*), an observation thought to be due to increased liver clearance of progesterone (*223*). It is unclear whether this observation extends to other steroids. Differences in maternal progesterone concentrations early in gestation are also associated with embryo survival (*141, 142*). Given these findings, the effect of nutritional restriction on progesterone and other steroid hormones (oestrogens and androgens) were considered important to measure in maternal plasma.

Steroids are also known to play key roles in fetal ovarian development with progesterone and oestrogen having been shown to be important in the breakdown of ovigerous cords and in follicle formation (*224-226*). Excess prenatal androgens have been shown to alter expression

of genes involved in germ cell proliferation and apoptosis (*227*). Further, effects of prenatal androgen excess on follicle development and function have been demonstrated in sheep (*228*).These effects are mediated directly by androgens and following their conversion to oestrogens. Expression of genes involved in steroid synthesis are evident in the fetal sheep ovary from as early as day 30 of gestation (*144*), and the ovaries are capable of producing steroids from this stage of development (*33*).Thus, steroid concentrations in fetal plasma were considered a possible mechanism by which gestational nutrition may affect fetal ovarian development and therefore important to measure in fetal plasma.

Restricted nutrition is known to have effects on the function of a number of organs in adult animals including kidney (*229*), liver (*230*), and the immune system (*231*), all of which have the potential to impact fetal development. Potential effects on major organ function can be evaluated by determining the metabolic status of both maternal animals and fetuses.

In non-pregnant adult ewes, hormone profiles are used to characterise oestrous cycles (*94*, *232*) and ovarian function (*94, 233, 234*). Therefore, hormone profiles in the female offspring from this study will be used to characterise aspects of the oestrous cycle and ovarian function in these offspring. Potential differences between ewes from restricted and maintenance dams may support the differences reported in Chapter 2 and offer insights into the mechanisms underlying the observed differences.

AMH was considered a key hormone to measure in offspring as it has widely been reported as an indicator of AFC, ovarian reserve, and fertility (*63, 235, 236*). Determination of AMH levels at the time of scanning and laparoscopy may support the differences in AFC reported in Chapter 2.

Progesterone concentrations in offspring were considered important, not only for its association with embryo survival (*141*), but also to characterise the oestrous cycles in the female offspring. While progesterone concentrations do not necessarily correlate with the OR (*237*), they do provide information on the functional integrity of the CL and cycle length.

FSH concentrations provide important information to characterise the ooestrous cycles and the ovaries in the offspring (*94*), particularly as FSH is sensitive to feedback from ovarian follicle-produced factors such as inhibin and oestradiol (*91*). Unlike FSH, the pattern of LH secretion is not influenced by feedback from inhibin and oestradiol. Therefore, peripheral blood concentrations of LH are considered an accurate reflection of hypothalamic GnRH secretion (*88*). Characterisation of the pattern of LH secretion in female offspring is therefore important to determine if alterations to the neuroendocrine system contribute to the observed increased indicators of fertility in these sheep.

Thus, the aims of this chapter were twofold:

Firstly, to determine if altered gestational nutrition affected concentrations of hormones and metabolic factors in maternal and fetal plasma which may contribute to the phenotype observed in female offspring.

Secondly, to characterise the oestrous cycles in female offspring from both groups, identifying changes which either contribute to, or result from the observed differences in OR and AFC.

3.2 Materials and methods

3.2.1 Theoretical basis of assays used in this study

3.2.1.1 Sandwich enzyme-linked immunosorbent assay for the determination of AMH

The concept of the AMH assay is illustrated and described in Figure 3.1. The AMH contained in the sample is incubated with, and binds to a known amount of a primary antibody (1°Ab, anti-AMH) which coats a tube or well. The amount of AMH which binds to the antibody is proportional to the amount of AMH present in the sample. A biotinylated secondary antibody (2°Ab) is added which also binds to the antigen. The enzyme horse radish peroxidase, conjugated to streptavidin (SHRP) is then added. The resulting biotin streptavidin binding results in the complex illustrated in Figure 3.1. Addition of a substrate (tetramethylbenzidine) results in the production of a coloured reaction product, which was measured at a wavelength of 450 nm using a multilabel plate reader (Victor3, Perkin Elmer, Waltham, MA, USA). The resultant colour intensities (optical densities or OD) are directly proportional to the concentration of AMH within the sample. The OD values are converted to concentrations by comparing the sample OD values to OD values generated from standards of known concentrations.

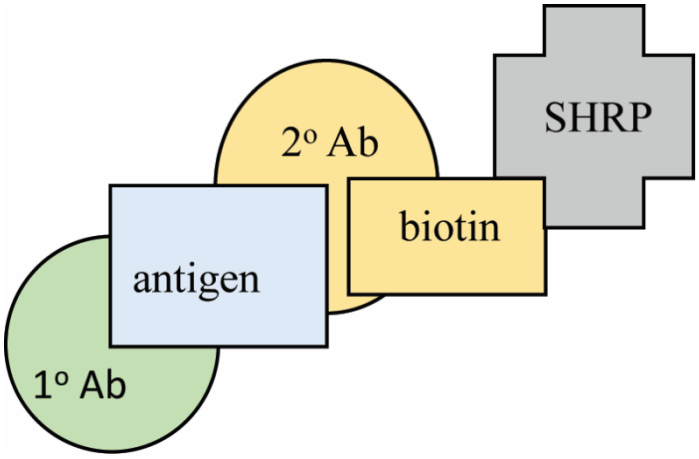

Figure 3.1 Basis of the AMH ELISA. Enzyme-linked immunosorbent assay (ELISA) plate wells are coated with an anti-AMH primary antibody (1°Ab). Antigen (AMH in a sample) first binds to a 1° Ab, the amount of binding is proportional to the antigen concentration. An anti-AMH secondary antibody (2°Ab) conjugated to biotin is added. This antibody binds to a different site (epitope) of the antigen. A streptavidin-horse radish peroxidase conjugate (SHRP) is added which binds to the biotin moiety of the 2°Ab. Addition of a substrate for the SHRP then produces a coloured reaction product, which is measured photometrically using a multi label plate reader. The amount of coloured reaction product produced and then measured is proportional to the concentration of antigen.

3.2.1.2 Competitive enzyme-linked immunosorbent assay for the determination of testosterone

The testosterone within a sample and a known amount of testosterone conjugated to the detection enzyme horse radish peroxidase (HRP), compete to bind to a 1°Ab which coats the tube. The relative amounts of sample testosterone and labelled testosterone that bind to the 1°Ab are determined by the sample concentration (as the concentration of the HRP labelled antigen is standardised). Following removal of excess sample and labelled antigens, only the labelled antigen-1°Ab complex produces a coloured reaction product. Thus, the OD of the coloured reaction product is inversely proportional to the concentration of sample antigen and is measured as described for the AMH ELISA (Section 3.2.1.1).

3.2.1.3 Competitive radioimmunoassay for measurement of progesterone and FSH

The concept of a competitive radioimmunoassay (RIA) is presented in Figure 3.2 using FSH as an example. FSH within a sample, 1° Ab, and a standard amount of radioactively (I^{125}) labelled FSH (tracer) are mixed together. Both labelled (I^{125}) and unlabelled (sample) FSH compete to bind to the antibody. The amount of each antigen bound is proportional to the concentration of sample antigen. A 2°Ab and polyethylene glycol are added to precipitate the bound and unbound 1°Ab, enabling separation of the excess FSH by centrifugation. The remaining precipitate is counted in a gamma counter (Wallac Wizard 1470, Perkin Elmer) which counts the radioactivity present in each tube and converts it to a concentration by comparing the sample counts to counts generated from a series of standards of known concentrations. The radioactivity counted (I^{125} labelled antibody-antigen complex) is inversely proportional to the concentration of sample antigen-antibody complex.

For progesterone assays, the primary antibody is pre-coated onto assay tubes, therefore following binding between progesterone and the antibody, unbound reagents are aspirated off and the dry tubes counted using a gamma counter.

3.2.1.4 Displacement radioimmunoassay for measurement of leptin and LH

In the displacement assays, the sample antigen (leptin or LH) and antibody are allowed to bind first. Following this binding, I^{125} labelled antigen is added that then displaces a proportion of the previously bound sample antigen. Following separation of unbound antigens by centrifugation, samples are counted as described for the competitive RIA.

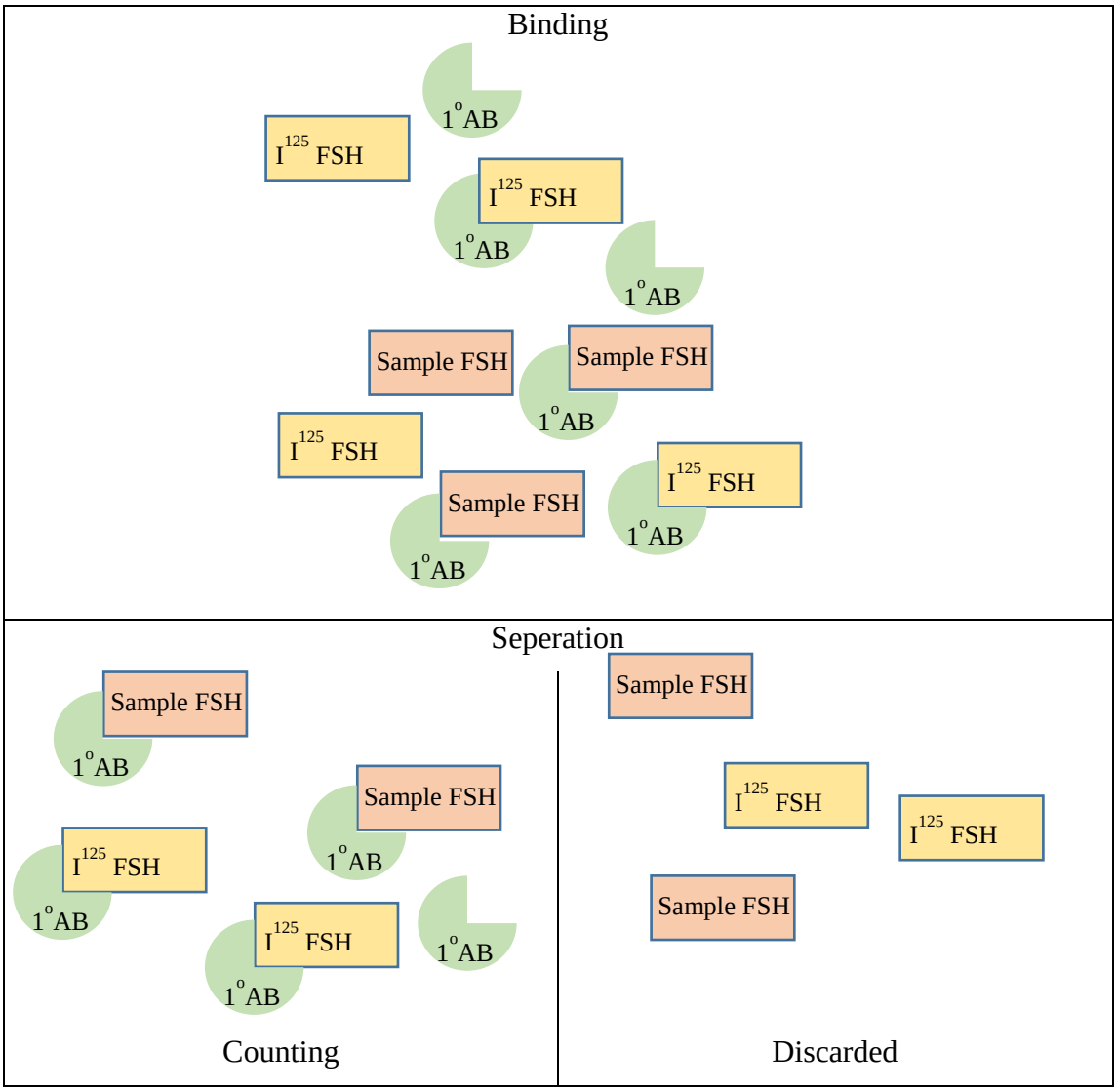

Figure 3.2 Concept of a competitive RIA (FSH). Binding occurs when sample containing FSH, labelled (I^{125}) FSH and 1°Ab are mixed. Both labelled and sample FSH compete to bind to the antibody. Separation is achieved by addition of a 2°Ab, followed by polyethylene glycol to precipitate all antibodies (bound and unbound). Centrifugation then separates the precipitated antibodies from excess antigens (labelled and labelled). Radioactivity is counted, with counts being inversely proportional to the concentration of sample FSH.

3.2.2 Assay procedures and indicators of assay performance

Samples of known concentration (QC samples) were interspersed throughout every assay. The purpose was twofold. Firstly, the variation between the QC samples within an assay measures the intra assay coefficient of variation (CV). QC samples are also used to measure the variation between assays (inter assay CV). The second purpose is to determine during validation whether assays are stable or if values drift from expected values. Assay drift is defined as significant variation between QC samples measured at the beginning of an assay and those at the end. Drift can be an issue in large assay formats.

Individual assays were structured to include approximately equal numbers of samples from both nutritional groups, distributed evenly throughout the assay. Duplicates of each sample were assayed and the mean value of the two duplicates was taken as the measured value. Where the CV between the two duplicates exceeded 15 to 20% (assay dependent), the sample was repeated in a subsequent assay.

A number of assay parameters are calculated to determine the quality of each assay and to assist in identifying problems should the assay not perform to expectations. Non-specific binding (NSB) represents the amount of antigen which binds to sites other than the 1° Ab, for example directly to the tube or well. This is measured by including tubes which contain no sample and no primary antibody. Following the standard assay procedure, the amount of radioactivity counted in these tubes represents the amount of labelled antigen which has bound to sites other than the 1° Ab. The counts obtained for NSB were subtracted from the counts for each sample tube so that the remaining counts represent only binding between the antigen and 1° Ab. For the ELISAs, NSB had been shown by the manufacturers to be negligible. As uncoated wells were not included in the kits, this could not be verified.

Binding represents the recognition efficiency between labelled antigen and antibody. For RIA, the most useful binding value is calculated by dividing the result from a zero standard by the total counts from the labelled antigen (tracer) alone. For ELISA, the value of the zero-standard divided by that of the maximum standard represents the most useful binding value. For both competitive and displacement assays providing that the NSB was low, low binding values generally indicate poor binding of the labelled antigen to the 1°Ab (often indicating damage to the labelled antigen as a result of a poor iodination when I^{125} is attached to the antigen). Poor binding of the labelled antigen to the 1°Ab results in low counts or low colour values, thereby

reducing assay sensitivity. Conversely, high values indicate high binding of the labelled antigen and therefore low binding of the antigen of interest, also resulting in low assay sensitivity.

Effective dose (e.g. ED50) was described as the concentration at which the compound of interest exhibits 50% of its maximum activity. In the case of an FSH RIA for example, it was the concentration of FSH which produces 50% of the counts of the zero standard (that is the maximum number of counts). An optimal ED50 value was roughly equivalent to the values obtained for the unknown samples. This places these values in the middle of the standard curve. Assay detection limit was the minimum concentration detectable by the assay. This was calculated as two standard deviations above the values obtained for the zero standard replicates. Functional detection limits can also be calculated from the plots of serially diluted samples with the detection limit being the lowest point where linearity in this plot was maintained.

3.2.3 Analysis of pulsatile secretion of LH

The LH assay was used to determine the characteristics of LH secretion over an 8-hour period. Two software packages, Dynpeak (*238*) and Autodecon (*239*), were trialled to analyse the data to determine variables such as pulse frequency, pulse amplitude, and basal secretion levels. Both packages utilise differing algorithms and have been used to generate published results in a variety of species (*240-242*). Both programs proved unsuitable for this dataset with false detection of peaks being prevalent. Given this issue, a standardised manual method was developed and applied to analyse the characteristics of LH secretion.

For each sheep, from the 33 samples measured over the 8-hour period, the average of the 10 lowest values was taken to be the basal secretion value. The standard deviation (SD) of the basal values was calculated. Values over 10 SD above basal values were considered a major peak, while values between three and 10 SD above basal values were considered minor peaks. To be considered a peak, at least one value had to fit the criteria where the proceeding value did not. These criteria eliminated points, such as indicated by the arrow in Figure 3.3, being described as a peak. Figure 3.3 shows a typical LH profile for an individual animal. For this example, Dynpeak indicated 4 peaks and Autodecon 5 peaks. Applying the manual criteria detailed above, 2 major peaks (red stars) and 1 minor peak (purple star) were detected. Variables (sum of all 33 values, average of all 33 values, basal level, number of major peaks, average major peak amplitude, number of minor peaks, average minor peak amplitude) were counted or calculated to give a complete picture of LH secretion.

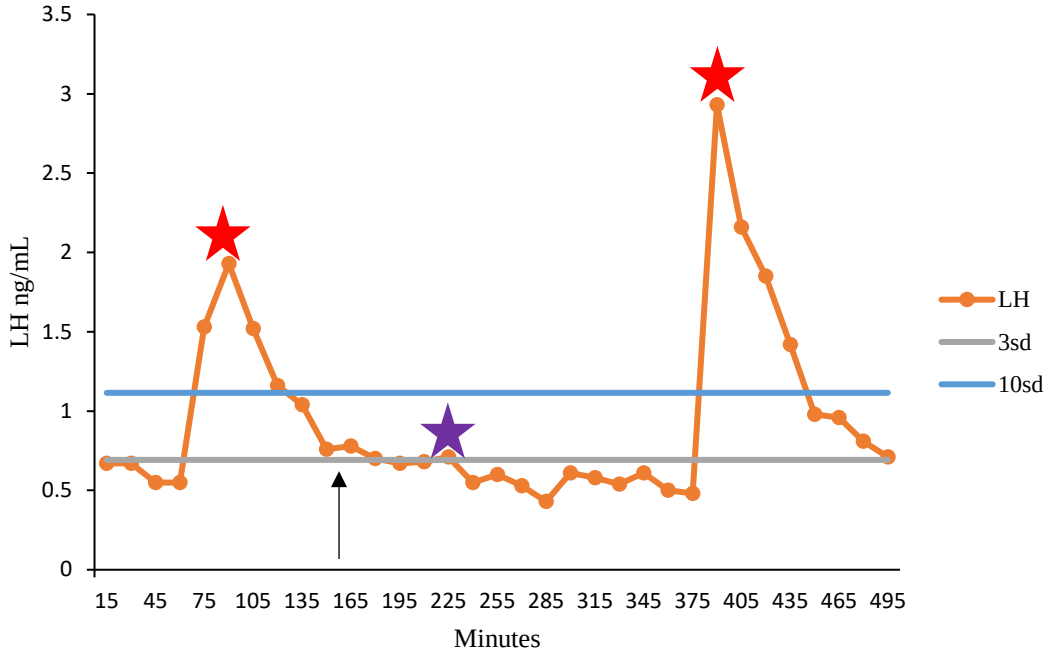

Figure 3.3 Peak selection applied to a typical LH profile. In this example, major peaks (> 10 SD above basal levels) are indicated by red stars. A minor peak (between 3 and 10 SD above basal) is indicated by a purple star. Arrow indicates potential false positive peak eliminated by applying the standard criteria. Basal secretion value for this animal was 0.59 ng/mL.

3.2.4 Source of assays

Leptin was measured using a Millipore multi-species RIA kit (Millipore, St Charles,MS, USA. Cat XL-85K). This kit uses an iodinated human leptin protein, and a guinea pig antibody raised against human leptin. The provided controls and standards are recombinant human leptin.

Testosterone was measured using the IBL Free Testosterone ELISA (IBL, Hamburg, Germany. Cat DB52181). The kit measures free testosterone which represents only 2 to 3% of total testosterone. The remaining testosterone in plasma is bound to factors such as steroid hormone binding globulin, and albumin (*243*). A polyclonal anti-testosterone antibody is used along with testosterone standards in human serum.

AMH was measured using the Equine Ovine AMH ELISA (Ansh Labs, Webster, TX, USA. Cat AL-115). Details of the antibodies used in this kit are proprietary knowledge. Equine standards are used.

Progesterone in maternal samples was measured using the Siemens coat-a-count RIA (Siemens Healthcare Diagnostics, Auckland, NZ. Product discontinued). For 19 month old female offspring, progesterone was measured using the IBL progesterone RIA (IBL, Hamburg, Germany. Cat MG12171). A polyclonal anti-progesterone antibody is used along with progesterone standards in human serum.

LH was measured using reagents obtained from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK-oLH-I-4). The kit uses a rabbit anti-ovine LH antibody (OLH-1), and a sheep anti rabbit second antibody. The method has been published previously (*232*).

FSH was measured using reagents obtained from National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK-oFSH RP2). The kit uses an anti-ovine FSH antibody, ovine FSH standards and a sheep anti rabbit second antibody. Standards were prepared from a highly purified ovine LH preparation (CY1085) The method has been published previously (*232*).

Liquid chromatography mass spectroscopy (LCMS) is considered the "gold standard" for determining steroid concentrations (*244*). LCMS was undertaken by the laboratory of Professor David Handelsman at the ANZAC Research Institute, Sydney, Australia. The method is described in Keski-Rahkonen et al (*245*).

Metabolic factors (albumin, beta hydroxyl butyrate, creatinine, triglycerides, Ca^{2+}, Mg^{2+}, and PO_4^{3-}) were measured by Gribbles Veterinary Labs (Mosgiel, NZ). All assays were photometric assays performed using a Roche-Hitachi Clinical Analyser. The laboratory is IANZ accredited to NZS ISO/IEC 17025:2005. All assays were performed using standard protocols which meet the accreditation criteria.

Detailed assay protocols are presented in Appendix A.

3.3 Results

3.3.1 Optimisation and validation of assays for sheep samples

3.3.1.1 Validation procedures

Assays for leptin, testosterone, progesterone (IBL kit) and AMH had not previously been used or validated for analysis of sheep samples. FSH and LH assays required new reagents, in accordance with standard laboratory procedures these reagents required validation before use with experimental samples. Each assay was validated using two methods. Firstly, serially diluted samples were assayed. Beginning with the measured value of an undiluted sample, the expected concentration of each dilution is plotted against the measured concentration of each dilution. The resulting plot should return linear results running parallel to the line generated by the standard curve. An example of these plots is presented in Figure 3.4 for the leptin assay.

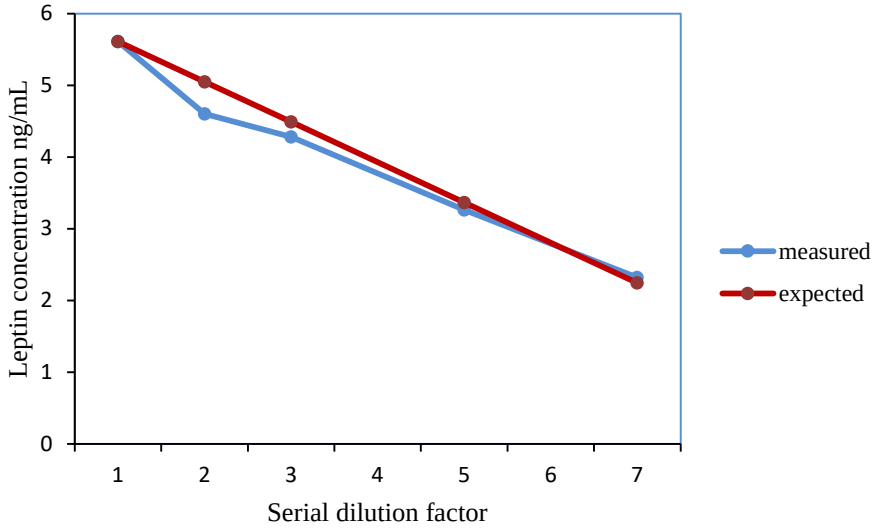

Figure 3.4 Leptin serial dilution. Shows minimal deviation (average 5%) between measured concentrations (blue) and expected concentrations (red). Experimental samples ranged between 3 to 6 ng/mL.

Deviations from the expected value (DEV) should remain < 10%. Secondly, spiked samples were used**.** For example, when 100 µL of a sample containing 2 ng/mL of FSH is added to 100 µL of a standard containing 8 ng/mL, the resulting 200 µL should have a concentration of 5 ng/mL ((2 + 8)/2). The measured value for this combined sample divided by the expected value is the % recovery, which should be close to 100%. Optimisation data including DEV recovery rates, inter assay CV, intra assay CV, and detection limits are presented in Table 3.1

3.3.1.2 Maternal leptin

For optimum assay performance, the manufacturer's instructions were modified as follows. 50 µL of standard and 50 µL of buffer were used as per instructions. Unknown samples and QC samples used 100 µL of plasma with no added buffer. The remaining steps were as recommended by the manufacturer. Following this modified protocol, serially diluted samples showed acceptable parallelism (Figure 3.4). Spiked samples showed recoveries between 91% and 104%. Inter-assay CV was 19%, intra-assay CV was 8.5% and the assay detection limit was 0.1 ng/mL.

3.3.1.3 Maternal testosterone

The assay used in this study posed a dilemma. The manufacturer's instructions state that samples should not be diluted as this process will alter the ratio of free:bound testosterone, effectively meaning that the assay cannot be validated by the standard criteria applied to the other assays used in this study. Experiments with samples serially diluted using a variety of diluents and incubation conditions demonstrated that this was the case, with linearity failing to be achieved. Given this, results from this assay are not presented in this thesis.

3.3.1.4 Maternal progesterone

The Siemens Coat-a-Count kit used for maternal progesterone samples was validated previously for use in sheep following the manufacturer's protocol. (*141*). In the current study, inter-assay CV was 12% and intra-assay CV was 5%. Assay detection limit was 0.1 ng/mL.

Table 3.1 Validation data for assays

Assay	Average DEV	Recovery rate (average)	Inter assay CV	Intra assay CV	Detection limit ng/mL	Assay accepted
AMH	42%	34%	n/a	n/a	0.5	no
Testosterone	50%	144%	n/a	n/a	n/a	no
Progesterone (IBL)	5%	102%	7.5%	9.5%	0.2	yes
Progesterone (Siemens)	Previously validated in sheep		12.0%	5.0%	0.1	yes
Leptin	5%	97%	19.0%	8.5%	0.1	yes
FSH	7%	96%	11.1%	9.2%	0.1	yes
LH	8%	94%	12.1%	7.8%	0.2	Yes

DEV indicates average deviation of measured concentration from expected concentration for serially diluted samples.

3.3.1.5 Female offspring AMH

Using an overnight primary antibody incubation at 4°C and subsequently following the manufacturer's recommended protocol, serial dilutions in assay buffer produced acceptable linearity and parallelism down to a concentration of approximately 0.5 ng/mL. Below this concentration, parallelism was consistently lost with deviations between measured concentration and expected concentration ranging from 16% to 65%. This indicates the effective lower limit of this assay is approximately 0.5 ng/mL. Almost 90% of samples measured from this study gave values below 0.5 ng/mL (most falling within the range of 0.05 to 0.1 ng/mL).

Additionally, recoveries from spiked samples within a range of concentrations from 0.04 to 5.0 ng/mL showed average recovery rates of 34%. Variations in sample volume and serial diluting samples in an in-house generated ovariectomised sheep plasma (as opposed to assay buffer), did not improve validation results. Given these issues, this assay was deemed unsuitable for use in this study.

3.3.1.6 Female offspring progesterone

The IBL kit had been developed and validated for human diagnostic use, and as such the recommended protocol had been designed for a rapid turnaround with the loss of some accuracy. Following the manufacturer's recommended protocol, three issues become apparent: serial dilution of samples or standards showed non-linearity and non-parallelism, spiked samples resulted in an unacceptable recovery rate of 168%, and significant assay drift was apparent with the internal control samples measuring 600% higher at the end of an assay (i.e. tubes 95 and 100) when compared to the start of an assay (tubes 19 to 24).

Validation procedures indicated that an overnight incubation at 4°C, rather than the recommended two hours at 37°C, produced acceptable linearity and parallelism from serially diluted samples (Table 3.1). Recovery rate from spiked samples ranged between 87 to 116% using the modified protocol. Assay drift was reduced by processing smaller numbers of samples in sections. Standard assay procedure is to add sample to all tubes (100 to 200 tubes), and then add labelled progesterone to all tubes. Dividing each assay into blocks of 20 tubes contributed to a reduction in assay drift with variation between start and end QC samples averaging 7.2% following this change to assay setup. Inter assay CV was 7.5% and intra assay CV was 9.5%. Assay detection limit was 0.2 ng/mL.

Comparisons between progesterone concentrations obtained for the high, medium and low QC samples for the Siemens and IBL progesterone assays are given in Table 3.2. While both assays have been validated, some variation was apparent between values obtained for the two assays particularly at lower concentrations (2 to 3 ng/mL) where most experimental samples from this study lie.

Table 3.2 Comparison between Siemens and IBL progesterone RIAs

QC sample	**High**		**Medium**		**Low**	
Assay	Siemens	IBL	Siemens	IBL	Siemens	IBL
Concentration (ng/mL)	8.24	7.81	5.28	5.52	2.20	3.12

Differences in values obtained for high, medium and low concentration QC samples are shown. Values are averages over 5 Siemens and 8 IBL assays.

3.3.1.7 Female offspring FSH

Following the standard protocol for this assay, serially diluted samples showed acceptable parallelism. Spiked samples returned recovery rates of between 93 to 99%. Inter assay CV was 11.1% and intra assay CV was 9.2%. Assay detection limit was 0.1 ng/mL.

3.3.1.8 Female offspring LH

Following the standard protocol for this assay, serially diluted samples showed acceptable parallelism. Spiked samples returned recovery rates of between 91 to 96%. Inter assay CV was 12.1% and intra assay CV was 7.8%. Assay detection limit was 0.2 ng/mL.

3.3.2 Assay results

3.3.2.1 Maternal metabolic factors

Concentrations of metabolic factors in maternal ewes along with normal ranges for these factors in sheep are presented in Table 3.3. In general, average concentrations of metabolic factors irrespective of gestational age or group were within the normal range for sheep. There were no significant effects of group or stage of pregnancy on the concentrations of beta hydroxybutyrate, Mg^{2+}, PO_4^{3-}, or triglycerides (GLM).

There were significant effects on albumin concentrations of stage of pregnancy ($p < 0.01$, GLM) and group ($p < 0.01$, GLM) and a significant stage of pregnancy x group interaction ($p = 0.018$, GLM). At day 55 of gestation, maternal concentrations of albumin were significantly lower in ewes on restricted nutrition compared to those on maintenance nutrition ($p < 0.01$, ANOVA).

For Ca^{2+} concentrations a significant effect of group ($p = 0.05$, GLM) and a significant stage of pregnancy x group interaction ($p = 0.038$, GLM) were noted. Concentrations were significantly lower in restricted ewes at day 55 compared to maintenance ewes ($p < 0.05$, ANOVA). Ca^{2+} concentrations in restricted ewes at day 55 were lower than the normal range for sheep, while all other Ca^{2+} concentrations were within the normal range.

There was a significant group effect on creatinine concentrations ($p < 0.05$, GLM) with no stage of pregnancy effect or stage of pregnancy x group interaction. Concentrations of creatinine were significantly higher in restricted ewes than maintenance ewes at both days 55 and day 75 ($p < 0.05$, ANOVA). The levels in restricted ewes were higher than the normal range for sheep at both stages of pregnancy.

Table 3.3 Maternal metabolic factors at day 55 and 75 of gestation

	Units	Normal Range	Day 55		Day 75	
			Maintenance	Restricted	Maintenance	Restricted
Albumin	g/L	26-37	33.3 ± 0.54*	28.6 ± 1.0	27.8 ± 0.4	27.3 ± 0.9
Beta-hydroxybutyrate	mmol/L	0-1	0.44 ± 0.04	0.45 ± 0.02	0.4 ± 0.05	0.4 ± 0.07
Calcium	mmol/L	2-3	2.26 ± 0.06*	1.97 ± 0.07	2.2 ± 0.1	2.2 ± 0.08
Creatinine	µmol/L	55-77	66.5 ± 2.5*	77.9 ± 3.7	76.3 ± 3.3*	87.5 ± 3.6
Magnesium	mmol/L	0.85-1.29	0.74 ± 0.03	0.77 ± 0.03	0.8 ± 0.03	0.8 ± 0.05
Phosphate	mmol/L	1.61-2.35	1.42 ± 0.04	1.27 ± 0.07	1.3 ± 0.1	1.5 ± 0.1
Triglyceride	mmol/L	n/a	0.16 ± 0.01	0.19 ± 0.01	0.18 ± 0.02	0.23 ± 0.04

Values are means and standard errors for 10 randomly selected animals for each group at each age. * indicates significant difference between groups at the indicated age ($p < 0.05$, ANOVA). Normal ranges are for non-pregnant sheep as ranges for pregnant sheep are not available.

3.3.2.2 Day 75 fetal metabolic factors

No significant effects of nutrition restriction on metabolic factors were observed in day 75 fetal plasma (Table 3.4). Normal concentration ranges for metabolic factors in fetal sheep is not currently available. When compared to maternal concentrations at the same day of gestation, fetal concentrations of electrolytes (Ca^{2+}, Mg^{2+}, and PO_4^{3-}) were similar. Albumin and creatinine were significantly less ($p < 0.01$, ANOVA) in fetal plasma, and the concentration of beta-hydroxybutyrate and triglyceride were significantly higher ($p < 0.01$, ANOVA) in fetal plasma.

Table 3.4 Concentration of metabolic factors in day 75 fetal plasma

Metabolic Factor	Units	Maintenance	Restricted
Albumin	g/L	12.8 ± 0.7	13.0 ± 1.0
Beta-hydroxybutyrate	mmol/L	0.13 ± 0.02	0.16 ± 0.02
Calcium	mmol/L	2.4 ± 0.3	2.6 ± 0.2
Creatinine	µmol/L	27.4 ± 1.6	26.4 ± 1.3
Magnesium	mmol/L	0.77 ± 0.05	0.77 ± 0.04
Phosphate	mmol/L	1.9 ± 0.1	1.9 ± 0.1
Triglyceride	mmol/L	0.30 ± 0.01	0.31 ± 0.02

Values are means and standard errors for 8 maintenance and 8 restricted fetuses. No significant differences were reported between groups (ANOVA).

3.3.2.3 Maternal leptin

A significant effect of stage of pregnancy was observed ($p < 0.01$, RMANCOVA), but no significant effect of nutritional group, and no group x day of gestation interaction (Figure 3.5) were noted on maternal leptin concentrations. The lack of both a group effect and interaction demonstrates that in this study, maternal leptin concentrations were not affected by the restricted nutritional regime at any stage of pregnancy examined. Given the role of leptin in appetite control and energy homeostasis, its production from adipose tissue, and the differences in body weight and BCS between groups this result was surprising and will be explored further in the discussion.

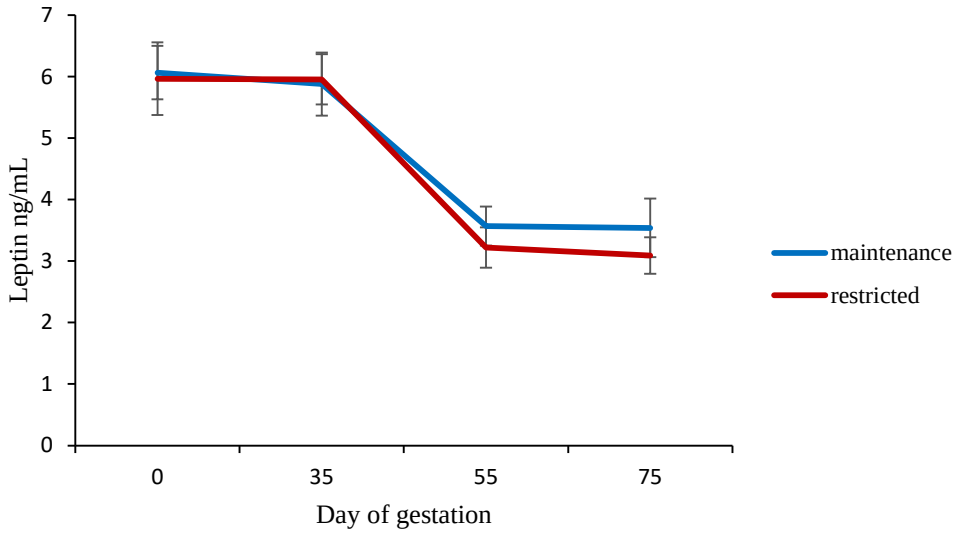

Figure 3.5 Maternal leptin concentrations during early gestation. Values are means and standard errors for between 8 and 20 samples per group, per day of gestation. A significant effect of day of gestation was detected ($p < 0.01$, RMANCOVA), but no effect of group or group x day of gestation interaction.

3.3.2.4 Maternal steroids

There was a significant effect of day of gestation ($p < 0.01$, GLM) and a significant effect of gestational nutrition ($p = 0.04$, GLM), with no group x day of gestation interaction ($p = 0.12$, GLM) on maternal progesterone concentrations. Animals exposed to restricted nutrition had increased progesterone concentrations compared to those animals on the maintenance diet (Figure 3.6).

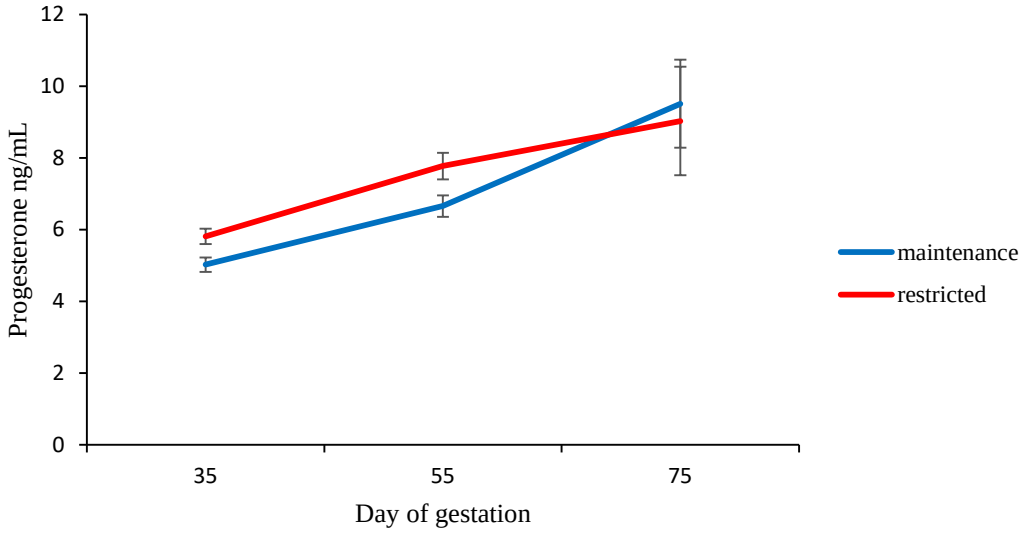

Figure 3.6 Maternal progesterone concentrations during early gestation. Values are means and standard errors for between 7 and 20 animals per day of gestation per group. A significant effect of both stage of pregnancy ($p < 0.01$) and group ($p < 0.05$) were detected with no stage of pregnancy x group interaction.

Maternal steroid concentrations determined by LCMS are shown in Table 3.5. LCMS was unable to detect testosterone, dihydrotestosterone, dehydroepiandrosterone, 3α-androstanediol, 3β-androstanediol, estrone, and androstenedione at either stage of pregnancy (day 55 and day 75). Oestradiol was detectable in 12 of 22 samples at day 55, and 14 of 15 samples at day 75. Where values were below the detection limit, expected values were generated using an iterative algorithm according to the method of Gleit (*246*). There were no differences between groups in the proportion of animals with detectable oestradiol concentrations. There was a significant effect of stage of pregnancy ($p < 0.01$, GLM) on oestradiol concentrations, but no effect of group and no stage of pregnancy x group interaction. Progesterone was detected in all samples. For progesterone concentrations, there was a significant effect of stage of pregnancy ($p < 0.01$, GLM), but no effect of group, and no stage of pregnancy x group interaction.

Table 3.5 Maternal steroid levels measured by LCMS

Hormone	Units	Detection limit	Day 55		Day 75	
			Maintenance	Restricted	Maintenance	Restricted
T	ng/mL	0.01	nd	nd	nd	nd
DHT	ng/mL	0.1	nd	nd	nd	nd
DHEA	ng/mL	0.05	nd	nd	nd	nd
3a diol	ng/mL	0.05	nd	nd	nd	nd
3b diol	ng/mL	0.05	nd	nd	nd	nd
E1	pg/mL	2.5	nd	nd	nd	nd
ASD	ng/mL	0.025	nd	nd	nd	nd
E2	pg/mL	0.125	0.28 ± 0.07	0.79 ± 0.24	1.69 ± 0.55	1.04 ± 0.22
P4	ng/mL	0.05	2.18 ± 0.17	2.81 ± 0.18	4.01 ± 0.53	3.60 ± 0.62

Values are means and standard errors for 22 samples at day 55 and 15 samples at day 75. nd = not detectable. T = testosterone, DHT = dihydrotestosterone, DHEA = dehydroepiandrosterone, 3a diol = 3α-androstanediol, 3b diol = 3β-androstanediol, E1 = estrone, ASD = androstenedione, E2 = oestradiol, P4 = progesterone.

3.3.2.5 Day 75 fetal steroids

Using LCMS, dihydrotestosterone, 3α-androstanediol, and androstenedione were not detected in the plasma of day 75 fetuses. No nutritional group differences were observed in the concentrations of progesterone, oestradiol, estrone, dehydroepiandrosterone or 3β-androstanediol (Table 3.6). Fetal concentrations of oestradiol were significantly higher ($p < 0.01$, ANOVA) than the corresponding maternal samples, while fetal progesterone concentrations were significantly lower ($p < 0.01$, ANOVA) than the corresponding maternal samples.

Table 3.6 Day 75 fetal steroids measured by LCMS

Hormone	Units	Maintenance	Restricted
Progesterone	ng/mL	0.24 ± 0.06	0.21 ± 0.02
Oestradiol	pg/mL	9.98 ± 1.29	8.17 ± 1.34
Estrone	pg/mL	73.07 ± 21.32	56.94 ± 6.48
DHEA	pg/mL	0.16 ± 0.02	0.16 ± 0.05

Values are means and standard errors for 14 samples. DHEA = dehydroepiandrosterone. 3β-androstanediol, testosterone, dihydrotestosterone, 3α-androstanediol, and androstenedione were not detected in these samples.

3.3.2.6 Female offspring progesterone

Progesterone concentrations on each day throughout a complete oestrous cycle of all 30 female offspring are presented in Figure 3.7. For this analysis, ovulation rate was included in the model. The results indicate a significant effect of day of the cycle ($p < 0.01$, RMANCOVA), a significant effect of gestational nutrition ($p = 0.038$, RMANCOVA), and no effect of ovulation rate ($p = 0.29$, RMANCOVA) on progesterone concentrations. There was no day of the cycle x nutritional group interaction ($p = 0.92$). Ewes from dams exposed to restricted nutrition during early gestation had higher progesterone concentrations when compared to ewes from maintenance dams. Multiple range tests (Bonferroni) indicate that these differences were apparent during the early stages of the oestrous cycle where progesterone levels are rising (Figure 3.7).

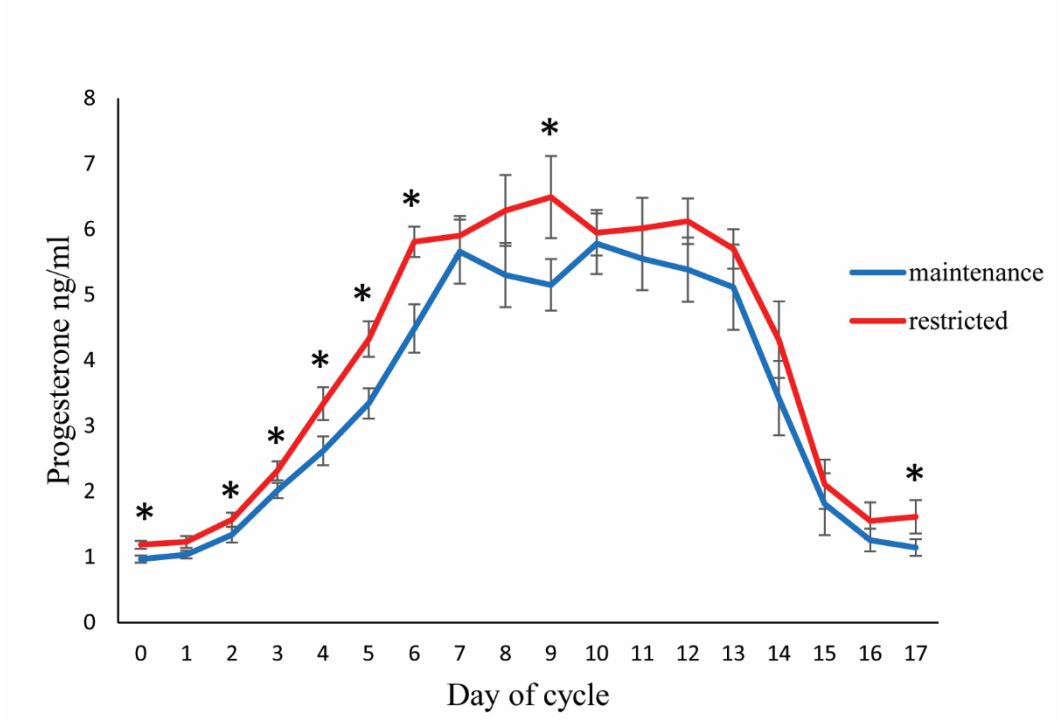

Figure 3.7 Daily progesterone levels throughout the oestrous cycle. Day 0 indicates day of mating. Values are means and standard errors for 13 restricted and 17 maintenance animals at 19 months of age. Significant day of the cycle and group effects were observed, both independent of the ewes' ovulation rate. * indicates significant between group difference at that day of the cycle by multiple range test ($p < 0.05$).

3.3.2.7 Female offspring LH

Analysis (ANOVA) of the pulsatile pattern of LH secretion using samples collected every 15 minutes over an 8-hour period did not identify any significant effects, on any of the variables analysed, attributable to the gestational nutrition regime of the ewes' dam (Table 3.7). This includes pulse frequency, pulse amplitude, basal secretion levels and total LH secreted over the 8-hour period.

Table 3.7 Characteristics of LH secretion in adult ewes

Variable	Units	Maintenance	Restricted
Sum of all values	ng/mL	31.2 ± 5.0	28.6 ± 2.3
Average all values	ng/mL	0.95 ± 0.2	0.87 ± 0.1
Basal level	ng/mL	0.54 ± 0.1	0.58 ± 0 .1
Number of major peaks	number	1.3 ± 0.5	1.9 ± 0.3
Average major peak amplitude	ng/mL	3.2 ± 0.5	2.4 ± 0.3
Number of minor peaks	number	0.9 ± 0.4	1.1 ± 0.2
Average minor peak amplitude	ng/mL	0.4 ± 0.2	0.4 ± 0.1

Values are means and standard errors for 10 maintenance and 10 restricted animals blood sampled every 15 minutes over an 8-hour period. No significant differences attributable to group were found for any of the variables.

3.3.2.8 Female offspring FSH

Using the same criteria (Section 3.2.3) and samples described for LH secretion, analysis did not detect significant FSH peaks. A typical FSH profile from an individual animal is shown in Figure 3.8.

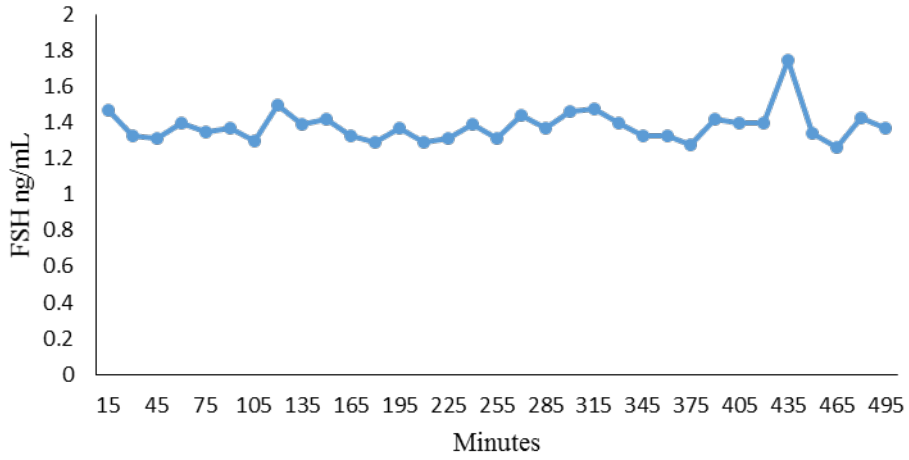

Figure 3.8 Typical FSH profile over 8-hour period. Typical FSH profile generated from samples collected every 15 minutes over an 8-hour period illustrating the lack of discernible, regular peaks.

A randomly chosen subset of 12 maintenance and 12 restricted animals were used to determine daily FSH concentrations throughout a complete oestrous cycle (Figure 3.9). There was no effect of nutritional group ($p = 0.2$, RMANCOVA), but an effect of day of the cycle ($p < 0.01$, RMANCOVA). However, a significant day of the cycle x group interaction ($p < 0.01$, RMANCOVA) was noted. The presence of a significant interaction indicates that the pattern of FSH concentrations throughout the cycle varies between groups. Multiple range tests (Bonferroni) demonstrated that ewes from dams exposed to restricted nutrition had lower FSH concentrations when compared to ewes from dams on maintenance nutrition at days 0, 2, 3, 4 and 17.

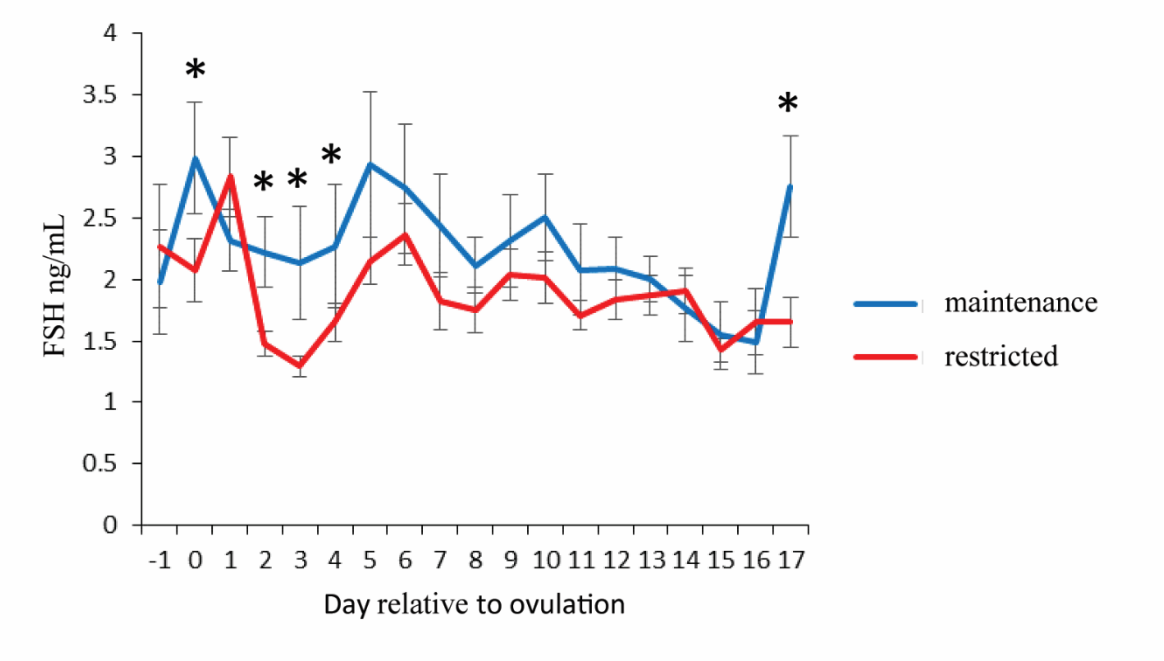

Figure 3.9 Daily FSH values throughout the oestrous cycle. Day 0 indicates day of mating. Values are means and standard errors for 12 restricted and 12 maintenance animals. While no overall effect of group was noted, a significant day of cycle effect and day of cycle x group interaction was noted. * indicates significant differences between groups at that age by multiple range test ($p < 0.05$).

3.4 Discussion

The results presented in this chapter indicate that the restricted nutrition regime not only resulted in lower maternal weights (Section 2.3.1), but also affected the metabolism of the pregnant ewes. Additionally, differences observed in hormone concentrations of 19-month-old female offspring expand on the 45% increase in AFC and 30% increase in OR observed in the animals from restricted dams compared to those from maintenance dams (Section 2.3.6). However, results of steroid and metabolic analysis of fetal plasma at day 75 have not demonstrated significant difference attributable to the restricted nutrition regime.

Results of the metabolic panel in maternal ewes follow a predictable pattern. Decreased albumin levels in restricted maternal ewes at day 55 of gestation are consistent with the literature. While albumin concentrations are often used as a test for liver function, low levels are associated with decreased nutrition as demonstrated in sheep by Caldeira and colleagues (*247*). Increased concentrations of creatinine reported in restricted maternal ewes have been reported previously in sheep on sub-maintenance feeding (*247, 248*). Increased creatinine concentrations are often an indication of decreased kidney function, however animals in the Caldeira study (*247*) and in the current study, showed no signs of significant kidney dysfunction. Given that chronic kidney disease is known to have negative fetal and maternal outcomes during pregnancy (*249*), the observation that all restricted animals that progressed to lambing did so normally, with no additional fetal loss, suggests that major issues related to kidney function had not developed in the animals on the restricted nutrition regime. While Caldeira and colleagues (*247*) reported differences in triglyceride concentrations, these differences were only evident when comparing animals with extreme differences in BCS (BCS 1 compared with BCS 4). The restricted animals in the current study lost < 1 BCS, therefore the absence of differences in triglyceride levels is in agreement with the Caldeira study. Similarly, the absence of differences in beta-hydroxybutyrate in the current study is also in agreement with the Caldeira study.

Of the electrolytes measured, only Ca^{2+} concentrations differed between the nutritional groups, with concentrations being lower in restricted ewes at day 55. Given the similarities in electrolyte levels between maternal and fetal plasma at day 75, one might expect to see a similar difference in day 55 fetal plasma. While this remains to be examined, should lower Ca^{2+} concentrations be found in plasma from restricted fetuses, this may have implications for fetal ovarian development, as Ca^{2+} is involved in a number of key cellular processes including cell death (*250*), and cell proliferation (*251*).

Three aspects of maternal leptin measurements made in this study are contradictory to what was expected. Firstly, no differences in leptin concentration were observed between nutrition groups from mating until day 75 of gestation. This result is somewhat surprising given the role of leptin in appetite control, and is in contrast to the study of Sosa which indicated that underfeeding produced differences in leptin levels in pregnant ewes (*252*) and the studies of Recabarren and colleagues (*83*), and also Delavaud and colleagues (*253*) in non-pregnant sheep. Secondly, leptin levels at day 55 were lower than those at day 35, whereas most studies, including those in sheep, report elevated leptin levels during gestation (*218*). Finally, following the change from restricted or maintenance feeding to ad libitum feeding (at day 55), leptin levels did not rise in either group as one may have expected.

In the sheep study by Sosa and colleagues, nutrition restriction began 14 days prior to mating with nutritionally induced differences in leptin levels apparent 9 days before mating. These differences were maintained until completion of the nutritional restriction at day 15 of pregnancy. Further, sheep were fed once daily with blood samples collected 1 hour prior to feeding and a nutritionally induced difference (maintenance compared to 50% of maintenance) in leptin levels was apparent.

In the current study, samples were collected approximately 1 hour following feeding. Thus, potentially a rapid leptin response to feeding in both nutritional groups may account for the lack of group differences. This concept may be supported by the fact that leptin secretion is episodic in nature as demonstrated in sheep by Recabarren and colleagues (*83*), although what regulates the episodic nature of leptin secretion is unknown. In humans following overnight fasting leptin continues to fall for 2 hours before rising to peak levels 8 hours following a meal (*254*). In contrast Weigle and colleagues conclude that food consumption does not increase plasma leptin levels. The response in sheep under extended maintenance and restricted nutrition is likely to be substantially different from those reports in humans, given both the restricted nature of the diet and the change in eating pattern from all day grazing. In the study of Recabarren and colleagues, nutritionally induced differences were reported in mean leptin levels, as well as the number and amplitude of pulses. However in contrast to the current study, Recabarren and colleagues performed their work on non-pregnant adolescent sheep, comparing maintenance to ad libitum feeding, and the timing and the relationship between feeding and blood sampling was not described.

Ehrhardt and colleagues reported an 80% rise in leptin levels from pre-breeding to mid-pregnancy in sheep (*218*) on a maintenance diet. In the current study however, a decrease of nearly 50% in leptin concentrations in both groups between day 35 and day 55 of gestation was observed. Concentrations then plateaued through until day 75. Similar to the current study the animals in the Ehrhardt study were fed twice daily although maintenance diets were introduced 20-40 days prior to mating. Thus differences in the length of maternal restriction between the two studies may explain some of the results. However, the study of Thomas and colleagues in pregnant adolescent sheep showed a pattern of maternal leptin concentration similar to the current study. While the study of Thomas showed increasing leptin concentrations for ewes on a maintenance diet from the day of mating until day 25 of gestation, a decline in leptin from day 25 was evident in the same ewes (*220*). In contrast ewes on a maintenance plus diet showed increasing leptin concentrations between day 50 and 70. Additionally the Thomas study showed changing from a moderate to a high diet at day 50 of gestation increased leptin levels while changing from a high to a moderate diet at day 50 decreased leptin levels.

Arguably the most plausible explanation for the surprising leptin results (and potentially the variation in results from previous studies) may relate to the assay used for measurements. This was the Millipore Multispecies leptin assay. This assay has published results in cattle (*255*), bats (*256*), voles (*257*), pigs (*258*), seals (*259*) and sheep (*260*). Further, the assay was validated for use with our samples, in that serial dilutions produced linearity, and spiking of samples gave good recoveries. While the study of Delavaud and collegues (*253*) demonstrated differences in leptin between nutritional groups, they also compared an ovine specific RIA with a multi-species RIA similar to the Millipore Multispecies assay. The multispecies assay returned values less than half those of the ovine specific assay. Further, the post-restriction comparison in leptin values was substantially less using the multispecies RIA and required using pre-trial values as a covariate to achieve significance.

In an interesting review Chilliard and colleagues raise some questions on the use of multispecies leptin assays in ruminants. Using a ruminant specific RIA, a rise in leptin levels was apparent immediately prior to parturition in cattle. Further, differences were apparent between high, medium and low nutritional groups in the same animals. Using a multispecies kit, the pre parturition rise in leptin was not apparent and differences between the medium and low nutrition groups were also not apparent (*261*). Thus for unknown reasons there appears to be an interaction between RIA tools and the physiological status of ruminants that potentially

precludes the use of nonspecific leptin RIAs in ruminants. While the reasons for this are unclear, it may be related to differences in ratio of free leptin to bound leptin (i.e. bound to a soluble receptor) (*261*) in animals under certain physiological conditions.

Thus, results for leptin in sheep during pregnancy and/or under differing nutritional planes varies considerably. Some factors appearing to contribute to this variation include the assay employed, the level of nutrition (over or underfeeding), timing of alterations to nutrition with respect to mating, and the age of animals. The potential effect of changes in feeding pattern and timing of blood sampling in relation to feeding may also contribute to variation between studies. Given that leptin is primarily produced in adipose tissue, differing fat composition of sheep breeds may have an effect on leptin levels and the response to nutritional challenges. Therefore, the breed of animals used and the metabolic status of animals prior to entering any nutritional experiment may also have an effect on leptin.

It is of interest that while the RIA results reported a significant group effect for maternal progesterone concentrations, the LCMS data did not report this difference. These seemingly divergent results most likely reflect the greater number of samples (and ages) used for the RIA. Further, if an ANOVA, as opposed to a GLM, is applied to the day 55 LCMS data, then a significant group difference is observed ($p = 0.012$). The differing outcomes between the two techniques highlights an important principle in interpretation of statistical data. Where statistical tests report no significant effects, this does not mean that values are the same. But, given the data, an effect was unable to be demonstrated. Given this, it appears that gestational nutrition may be affecting maternal progesterone concentrations.

Similar differences in progesterone concentrations observed using RIA in the current study have been reported in previous studies. These differences are thought to be, at least to some extent, due to nutritionally induced differences in blood flow through the liver. This results in differences in the metabolic clearance of progesterone (*262*). Differences in maternal progesterone concentrations are unlikely to have direct effects on fetal gonadal development. While a close association exists between progesterone metabolites in fetal and maternal circulations, there is no such correlation in progesterone concentrations (*263*). However, the observed differences in progesterone concentrations in maternal plasma do illustrate that the restricted nutrition does have a metabolic impact on the maternal ewes. These differences may provide a useful tool in future studies to measure the impacts of different nutritional regimes.

Rasby (*264*) reports elevated oestradiol levels late in gestation following early gestational nutritional restriction. There is no evidence from the current study to suggest nutritionally induced differences in maternal oestradiol concentrations. Using LCMS analysis, oestradiol and progesterone were detectable in maternal plasma at days 55 and 75 of pregnancy, with oestradiol detectable in only a proportion of animals. Progesterone concentrations measured by LCMS were approximately three fold higher than those obtained by RIA. However, the same pattern was observed with restricted animals having higher concentrations than maintenance animals.

Analysis of steroid concentrations in day 75 fetal plasma using LCMS did not detect any differences between nutritional groups. While the fetal sheep ovary does produce steroids from relatively early in gestation (*33, 144*), the high levels of estrone measured in fetal samples indicates that these steroids are predominantly placental in origin (*265*). The observation that maternal progesterone concentrations are higher than fetal concentrations, whereas fetal oestradiol concentrations are higher than those in the maternal system, likely reflect the source and regulation of these steroids. At day 75 of gestation, most maternal progesterone is produced by the CL of the ovary. Only at day 90 does the placental progesterone contribution reach parity with the CL (*266*). Placental oestradiol production is regulated by FSH, whereas progesterone production is regulated by prostaglandins (*267*). The high levels of oestradiol in fetal plasma compared to maternal plasma at day 75 may represent an earlier up-regulation of placental oestradiol compared to progesterone. The absence of correlations between maternal and fetal progesterone and oestrogen(s) concentrations demonstrates partitioning between maternal and fetal steroids in line with previous studies (*268, 269*). While steroids are known to play critical roles in fetal ovarian development (*146, 226, 270*), there is no evidence that differences in steroid concentrations contribute to the findings observed thus far. However, analysis of fetal plasma at day 55 of gestation, at the completion of the restricted nutrition period, would have provided a more complete picture. Further, the data does not discount autocrine effects of ovarian produced steroids.

A number of studies show a link between high progesterone concentrations and increased embryo survival, with this link particularly evident early in gestation (*141, 142*). The significant early rise in progesterone concentrations, evident in adult ewes from restricted dams, suggests that the increased OR and AFC observed in these animals (Section 2.3.6) has the potential to be accompanied by increased embryo survival. This would add another increased indicator of fertility to these animals. The early rise in progesterone in ewes from restricted dams is

independent of the OR rate in these animals, an observation also consistent with a likely increase in embryo survival in ewes from restricted dams.

The observed pattern of FSH secretion throughout the oestrous cycle in this study follows the typical pattern observed in sheep (*94*). Transient rises in FSH occur throughout the cycle, with these rises corresponding to the emergence of follicle waves (*207*). The most significant differences between the ewes from restricted or maintenance dams occur following the emergence of the first follicle wave (days 3 and 4). The first follicle wave is known to be especially active in the production of oestradiol and inhibin (*271*), both inhibitors of FSH secretion. Given that more antral follicles are observed in adult ewes from restricted dams, the inference is that the lower concentrations of FSH observed at the beginning of the oestrous cycle are the result of increased feedback from higher levels of follicle produced oestradiol and/or inhibin.

Sheep generally exhibit three or four follicle waves per cycle. Sheep with four follicle waves per cycle have both elevated progesterone levels, and lower average FSH levels throughout the cycle (*94*), both of these characteristics being evident in ewes from restricted dams. Close examination of FSH concentrations throughout the oestrous cycle (Figure 3.9), shows a rise in FSH concentrations in ewes from maintenance dams, indicative of the emergence of a new follicular wave, on days 0, 5 and 10. In ewes from restricted dams, increases in FSH concentrations are apparent on days 1, 6 and 9, with an additional small rise on day 14. While this may be interpreted as ewes from restricted dams having four follicle waves per cycle as opposed to three in maintenance animals, more intensive ultrasound scanning of follicle populations throughout the cycle is needed to confirm this.

Analysis of FSH from serial blood samples, collected every 15 minutes over an 8-hour period, did not show the presence of clearly distinguishable peaks (see Figure 3.8). This is a common feature of FSH secretion. While FSH pulses can be detected in hypophyseal portal blood (*272*), they are difficult to detect in peripheral plasma. This is thought to be due to the long circulating half-life of FSH and additional secretion of FSH pulses between observed pulses of GnRH (*273*). Additionally, multiple isoforms of FSH with differing half-lives further complicates detection of pulses (*274*). Thus, no conclusions regarding effects of dam nutrition on the pulsatile pattern of FSH secretion in female offspring can be drawn from the current study.

No differences were observed in the pattern of LH secretion. The finding of low pulse frequency at this stage of the cycle (1 to 2 pulses over the 8-hour period) is in line with expectations (*275*).

Progesterone and oestradiol feedback maintain this low pulse frequency thereby preventing final follicle maturation early in the oestrous cycle. With the pattern of LH secretion more accurately reflecting the pattern of GnRH secretion by the hypothalamus (*88*), the lack of differences between the groups is consistent with the concept that neuroendocrine control of follicle development in the female offspring has not been directly affected by the alterations to the dams gestational nutrition.

Two assays trialled in this study could not be validated to a level where the results were acceptable. The free testosterone assay specifically stated that samples should not be diluted, thereby making the key serial dilution validation impossible. During validation procedures, results from a number of maternal samples demonstrated free testosterone values of 11.3 pg/mL at day 55 and 4.3 pg/mL at day 75. These values are significantly higher than the manufacturer's guidelines for human females (95% percentile 0.2 to 1.3 pg/mL) and are similar to expected values for human males (95% percentile 2.6 to 9.8 pg/mL). Given that the assay measures only free testosterone which accounts for < 5% of total testosterone (*243*), this would suggest total testosterone values of around 214 pg/mL at day 55, and 81 pg/mL at day 75. However, total testosterone measured by LCMS, considered the gold standard for steroid measurement, could not be detected in the same samples. Given the detection limit of 10 pg/mL for the LCMS method, considerable doubt exists as to validity of the free testosterone assay for use in sheep.

The AMH assay, despite being marketed as an equine/ovine assay, could not be validated in our laboratory. The sensitivity of the assay, as indicated by serial dilutions, did not extend into the concentration range where most samples fell. Measurement of AMH has been problematic in most ruminants with the issues being best described by Arouche and colleagues(*276*). Arouche contends that to gain the sensitivity required to measure ruminant AMH, antibodies need to be produced against a ruminant AMH protein. Additionally, the epitope (the antigen site recognised by the antibody) may well be critical in determining binding and therefore the sensitivity of the assay. With the association between ovarian reserve, AFC, fertility, and AMH being widely accepted, determination of AMH concentrations in the female offspring from this study would add considerable strength to the current findings. Therefore, development of an appropriate assay such as the BOC ELISA described by Arouche will continue to be explored.

Overall, the results are consistent with the restricted gestational nutrition regime affecting progesterone concentrations in the pregnant ewes. Limited studies in fetal plasma have yet to identify any differences between hormone or metabolite concentrations in fetuses exposed to

either restricted or maintenance gestational nutrition. Differences in progesterone and FSH, but not LH, in adult female offspring suggest that the differences in indicators of fertility are ovarian driven. Whether these ovarian driven differences are a consequence of altered fetal ovarian development will be the focus of Chapters 4, 5, and 6.

Chapter 4 . Fetal Ovarian and Germ Cell Development

4.1 Introduction

The current study offers the rare opportunity to examine fetal ovarian development in the same cohort of animals in which gestational nutrition has been shown to affect indicators of fertility in female offspring. While most other studies have highlighted the negative effects of restricted gestational nutrition, in the current study, multiple indicators of increased fertility (OR, AFC, progesterone concentrations) have been observed in female offspring of dams exposed to restricted gestational nutrition. The underlying assumption, from both previous studies and the current study, has been that alterations to gestational nutrition has affected fetal ovarian development. The effects on ovarian development result in changes to the structure and/or function of the postnatal ovary, with the consequence of these changes being the observed alterations to fertility indicators. Of the many processes in the developing ovary, germ cell development seems the most likely aspect to be affected. Germ cell development involves a range of developmental processes (mitosis, meiosis, apoptosis, follicle formation and growth), is subject to complex regulation, and also has the potential to affect the ovarian reserve which is a key determinant of adult fertility (*63*).

Reported effects of gestational nutrition on fetal ovarian/germ cell development in sheep are not without precedent. Borwick and colleagues reported increased numbers of germ cells in day 42 and day 62 fetal ovaries from nutritionally restricted dams when compared to overfed dams (*172*). While it is difficult to determine whether this effect is from underfeeding or overfeeding, it does none the less establish a link between gestational nutrition and germ cell development. Asmad showed that ad libitum feeding during gestation increased numbers of primordial follicles, but decreased numbers of primary follicles at day 100 of gestation (*159*). The Asmad study is confounded by the differing weights of the ewes at mating, but it also highlights the link between gestational nutrition and germ cell development. Lea demonstrated that underfeeding from mating until day 30 of gestation reduced germ cell staining for the proliferation marker Ki-67 at day 65 of gestation (*171*). The same study also reported a prospective relationship between underfeeding from day 30 to day 65, and increased expression of the pro-apoptosis regulator *BAX* in germ cells.

Links between gestational nutrition and the ovarian reserve in female offspring in species other than sheep have also been reported. In cattle, Mossa and colleagues demonstrated that nutrition

restriction early in gestation decreased the ovarian reserve in female offspring (*65*). Similarly in the rat, offspring born to under fed mothers throughout gestation showed decreased primordial, secondary, and antral follicle numbers (*169*).

In addition to effects on germ cell development, restricted gestational nutrition has been shown to have effects on other cells in the sheep fetal ovary. Restricted nutrition from 20 days prior to mating through until day 135 of gestation, significantly reduced proliferation rates of follicular granulosa cells (*176*). The study of Lea and colleagues (*171*) showed under feeding from the day of mating until day 30 of gestation significantly increased granulosa cell proliferation at day 110 of gestation. This study also showed restricted nutrition from either mating until day 110, or from day 65 until day 110, also increased granulosa cell staining for *BAX* at day 110 of gestation (*171*).

While some studies have shown effects of gestational nutrition on the hypothalamic-pituitary-gonadal (HPG) axis, the timing of nutritional restriction in these studies commences later than the regime employed in the current study (*170*, *277*). This timing is consistent with the work of Thomas (*278*), who showed the first appearance of pituitary cells positive for the FSHβ subunit at day 100 of gestation. Thus, complete hypothalamic-pituitary development in the sheep fetus occurs well after the restriction regime employed in this study ended. Given this, any effects on the reproductive system produced by the nutritional regime used in this study are likely to be direct effects on the developing ovary, and not as a consequence of effects at the level of the developing hypothalamus or pituitary. This contention is supported by the lack of significant differences in the pattern of LH secretion in adult female offspring reported in Chapter 3.

The aim of the work described in this chapter was to determine whether the restricted gestational nutrition regime employed had affected the morphological development of the fetal ovary. The focus of this work is targeted at quantifying germ cell development, particularly the key processes of proliferation, autophagy, and apoptosis. These processes largely determine the size of the ovarian reserve.

4.2 Materials and methods

4.2.1 Histology

Following overnight fixation in 4% paraformaldehyde, ovaries were rinsed overnight in 70% ethanol. The ovaries were then processed and embedded in paraffin following a standard processing schedule (Appendix B). To facilitate subsequent immunohistochemistry, a low melting temperature wax (Shandon Paraplast X-tra, melting point 50 to 54°C. Thermo Fisher Scientific, Auckland, NZ. Cat 503002) was employed for processing and embedding of all samples. Ovaries were sectioned on a rotary microtome (Leica RM2135) at 5 µm with all sections retained. Every 10^{th} and 11^{th} section was stained with Haematoxylin and Eosin (Appendix C) for stereology and assessment of gross morphology (presence/absence of mitotic germ cells, meiosis, apoptosis, ovigerous cords, and ovarian rete).

4.2.2 The basis of stereological techniques applied in this study

Previous studies examining germ cell development in fetal ovaries from nutritionally restricted dams have utilised cell densities (or number of cells per unit area) as an indicator of germ cell numbers. While in general this is a reasonable approach, it fails to account for two sources of error. The first of these potential errors related to the reference volume. Using cell densities assumes that the volume of the ovary containing germ cells (or the ovarian cortex) is similar between the two groups being compared. This method is often justified on the basis that ovarian weights are similar between the groups. However, as germ cells are only located in the ovarian cortex, differences in the development or volume of the ovarian cortex may not necessarily be reflected in ovarian weights. Secondly, a size bias is introduced. The larger the cells being counted, the more likely they are to appear in an individual histological section (*279*).

To account for these type of errors, the current study uses stereological methods to provide accurate, unbiased estimates of total germ cell numbers within the ovary (*279*). The method first estimates the volume of the ovary or ovarian cortex using Cavileiris' principle. Cavaleiri estimates the area of interest by counting points which overlay these areas, on regularly spaced sections. The areas are converted to volumes by multiplying the total area by the distance between the sections counted. For germ cell number, the ovarian cortex was used as the reference volume as all germ cells which go on to produce follicles are located in the cortex (*34*). The number of cells per unit volume of ovarian cortex is then estimated using a physical dissector. The physical dissector involves overlaying a counting frame of known area over an image containing ovarian cortex. Cells of interest are counted within the frame which were not

present in the same area of the preceding section. Combining the two values gives a reliable estimate of the number of cells per ovarian cortex (or effectively the ovary, as developing germ cells are only located in the ovarian cortex) (*38, 279*).

These methods take account of any differences in cortical volume between the two groups being examined, and potential differences in germ cell size between the groups. Stereological methods have been previously used to examine fetal ovary development in sheep (*38*).

In all stereological studies, the sampling strategy employed is critical to produce unbiased, accurate estimates. The sampling strategy used in the current work was based on previous stereological studies of fetal ovaries (*38, 280*). Sections were chosen using an independent uniform random sampling strategy (i.e. systematically from a random start). Areas to count were selected using a double random number method. Initially, a computer generated random number between 1 and 12 was generated, with the area selected to count being where the random number appears on a clock face. A second random number between 1 and 3 was generated to select the zone to count within the ovarian cortex, relative to the ovarian surface. This principle is illustrated in Figure 4.1, with an ovary inserted inside a clock face. Where the first random number is 8 and the second number is 2, the area to be counted is represented by the red square.

When applying stereological techniques (both Cavaleiri and dissector) to quantify cells within an organ, the important variables to consider are the number of sections used, and the actual number of cells counted in the nucleator volume. Gundersen and Jensen recommend that provided the sampling strategy is appropriate, then counting more than 200 cells does not improve the accuracy of the estimation (*281*). In the current study, between 80 and 120 germ cells were counted in each day 55 ovary, and between 100 and 160 germ cells in each day 75 ovary. The physical dissector was applied to an average of 10 section pairs for each day 55 ovary, and 16 section pairs for each day 75 ovary.

Each germ cell counted was classified as either an oogonia or oocyte based on morphological criteria (*38*). Oogonia, were smaller than oocytes, possessed an intact nucleus with a smooth well defined border and a heterogeneous staining pattern. Oocytes (germ cells that had entered meiosis) had either a homogeneous nucleus with an irregular border, or no nuclear membrane but with thread like chromatin material. Oocytes were, on occasions, enclosed in isolated follicles as defined by the criteria of Smith and colleagues (*38*).

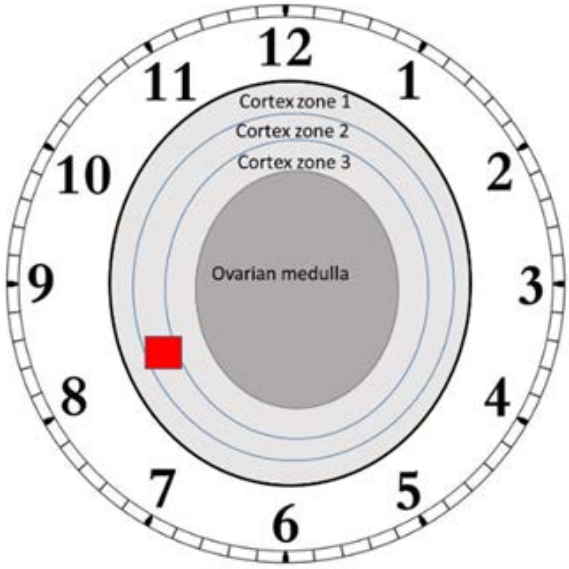

Figure 4.1 Principle of area selection for germ cell counting. Two random numbers are generated. The first number was between 1 and 12, which corresponds to the numbers on a clock face and indicates the area of the section to be examined. The second number was between 1 and 3 and corresponds to the zone of the cortex to be examined. Where the first number is 8 and the second number is 2, then the area to be counted is represented by the red square.

4.2.3 Application of stereology to fetal ovaries

The equation used for Cavaleiris' volume estimations was

$$V = (A)^2 \times \sum P \times D$$

V = reference volume (for this study the ovarian cortex)

A = the area of each point (for this study 0.04 mm^2 allowing for magnification)

$\sum P$ = the total number of points counted

D = distance between sections (for this study every 50^{th} 5µm section was counted, therefore D was 250 µm, or 0.25 mm)

For the physical dissector, firstly the volume used for counting on each pair of sections (nucleator volume) is calculated using the equation

$$v = (a \times d) \times n$$

v = volume in which cells were counted

a = area of the counting frame (for this study 0.12 mm^2 allowing for magnification)

d = distance between adjacent sections counted (for this study 5 µm)

n = number of counting frames examined.

The number of cells per ovary was then calculated using the equation

$$N(gc) = (V/\sum v) \times n$$

N(gc) = total number of number of germ cells

V = reference volume (for this study, the cortex volume)

$\sum v$ = sum of nucleator volumes used

n = total number of cells counted within the nucleator

4.2.4 Basis of immunohistochemistry for identification of germ cell proliferation, autophagy and apoptosis

Immunohistochemistry (IHC) for both proliferation and autophagy utilised the Dako Envision™ system (Dako Envision™ + Dual Link system-HRP DAB+. Cat K4065. Dako North America, Carpenteria, CA, USA). The envision system eliminates the use of biotin and streptavidin in the IHC process, thereby simplifying the protocol, reducing background staining, and increasing sensitivity. This is achieved by directly conjugating the HRP enzyme and secondary antibody (goat anti-mouse) to a dextran polymer. The concept of the Envision system is illustrated in Figure 4.2.

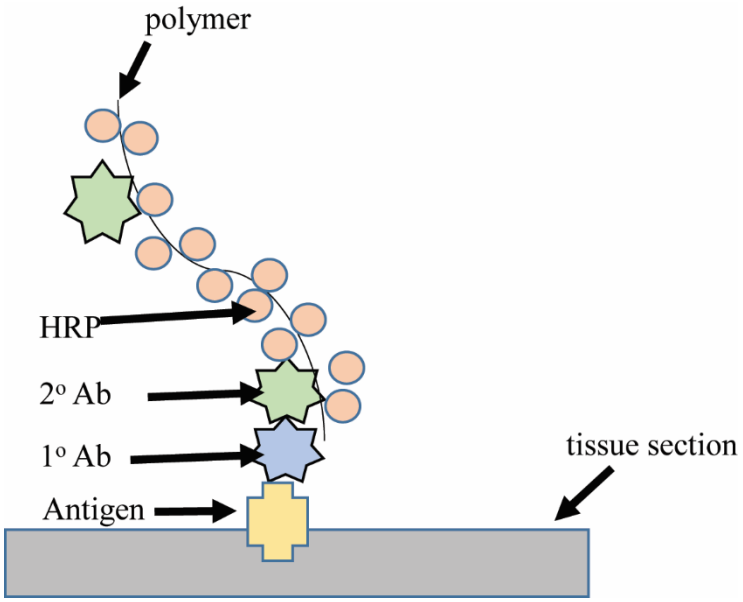

Figure 4.2 Concept of the Dako Envision system. Following incubation and binding of the 1° Ab (Ki-67 for proliferation or MAPLC3 for autophagy) to the antigen in the section, the polymer (conjugated to the 2° Ab and HRP) is added. The 2°Ab then binds the 1°Ab. Addition of the HRP substrate produces a coloured reaction product which can be visualised under the microscope.

Proliferative cells were identified using immunohistochemistry (IHC) against Ki-67, a protein present in the nuclei of some cells in the G_1 phase of the cell cycle, and in all cells in the S, G_2 and M phases of the cell cycle. Quiescent cells in the G_0 phase do not express the Ki-67 antigen. This makes Ki-67 a reliable means of assessing the fraction of proliferating cells within a large population of cells (*282*).

Autophagy, often referred to as type 2 programmed cell death, is a process where cell components are degraded within membrane bound vesicles. IHC detection of MAPLC3 (involved in expansion and fusion of autophagosomal membranes) used in the current study, is the most effective and widely accepted marker, largely because of its specificity compared to other autophagy markers (*283*).

Detection of apoptotic cells used the Terminal deoxynucleotidyl transferase dUTP nick end labeling reaction (TUNEL). TUNEL detects DNA fragmentation present during the final stages of apoptosis. dUTPs conjugated to biotin are enzymatically incorporated into the fragmented DNA and subsequently visualised using a streptavidin-HRP based detection system. Positive control sections were generated by incubation with a nuclease enzyme, supplied with the kit, and performing the labelling reaction. The nuclease enzyme generates breaks in the DNA in the section, and following completion of the protocol, all cells in the nuclease treated sections should stain positive indicating nicked DNA, mimicking apoptosis. Negative controls were created by omitting the terminal deoxynucleotidyl transferase (TdT) enzyme from the labelling reaction. This enzyme catalyses the insertion of biotinylated dUTP into breaks in the DNA. Following completion of the protocol, these sections should exhibit no staining.

All IHC protocols were preceded by deparaffinisation followed by hydration steps. Following IHC staining, sections were dehydrated and cleared. Full IHC protocols are presented in Appendix D.

4.2.5 Immunohistochemistry for proliferating cells (Ki-67)

Antigen retrieval was performed at 95°C in 10mM EDTA pH 8 for 20 minutes (2 x 10 minute incubations). After cooling for 20 minutes, a 5-minute peroxidase block was performed with 0.3% hydrogen peroxide in methanol at room temperature (RT). Incubation with the 1°Ab (1:50 dilution in antibody diluent, Dako North America, Carpenteria, CA, USA. Cat S3022) was for 30 minutes at RT, followed by a 30-minute RT incubation with the peroxidase labelled polymer (supplied ready to use). Following a 10-minute RT incubation with 3, 3 diaminobenzidine (DAB)/chromogen/substrate solution, cells were counterstained with 2% aqueous methyl green for 30 seconds. The primary antibody used was a monoclonal mouse anti-human Ki-67 (type IgG1), clone MIB1 (Dako, North America, Cat M7240). The negative control antibody used was Dako negative control mouse IgG1 (Dako, North America. Cat X0931). Sections of mature sheep ovaries containing numerous ovarian follicles, fixed and processed following a similar protocol, were included as positive control sections.

4.2.6 Immunohistochemistry for autophagy

Antigen retrieval was performed at 95°C in 10mM citrate buffer pH 6.0, for 10 minutes. Subsequent steps were identical to those used for proliferation (Section 4.2.5). The primary antibody used was mouse anti MAPLC3β monoclonal antibody (Santa Cruz Biotechnology, Dallas, TX, USA. Cat sc271625) of the IgG2b isotype at a 1:100 dilution. The negative control antibody used was Dako negative control mouse IgG2b (Dako, North America. Cat X0944).

4.2.7 Immunohistochemistry for apoptosis (TUNEL)

Apoptotic cells were detected using the Trevigen TACS 2 TdT-DAB In situ Apoptosis Detection kit (Trevigen, Gaithersburg, MD, USA. Cat 4810-30-K). The kit was optimised for sheep histological sections following the manufacture's guidelines. Sections were then incubated in a 1:50 proteinase K solution for 15 minutes at 37°C (as per the manufacturer's instructions). Following a 5-minute (RT) endogenous peroxidase block in 0.03% hydrogen peroxide in methanol, the labelling reaction was performed at 37°C for 60 minutes. The labelling reaction mix required the addition of one of three cations. Manganese at the recommended dilution was shown to produce optimal results and was included in all assays. Sections were subsequently incubated with SHRP complex for 10 minutes at 37°C. Following a 4-minute RT incubation in DAB, sections were counterstained with 2% methyl green.

4.2.8 Quantification of immunohistochemistry

While stereology is appropriate for total germ cell numbers, the frequency of positively stained cells using IHC is relatively low, making the application of stereological methods to these samples impracticable. For these samples, either staining intensity or positive cells per area of ovary were used as an indicator of their relative number.

Sections for IHC from each ovary were randomly selected based on computer generated random numbers, and the number of positive staining germ cells in each section were counted. To estimate the area of the ovary on the section, a 100 µm grid was superimposed over the image and the number of points over the ovary were counted. Three to four sections from each ovary were counted, with the values for the sections from an individual ovary being averaged prior to analysis. The number of positive staining cells were then divided by the number of points, with the result being expressed as the relative number of proliferating germ cells per unit area of ovary (volume from Cavaleiri Section 4.2.3).

For autophagy, due to the widespread pattern of staining, an alternative strategy was employed to quantitate positive staining cells. Three micrographs were taken from randomly selected fields for each fetal ovary. Micrographs were taken at the same magnification (250x) under identical illumination conditions. The micrographs were assessed by three independent persons, with each micrograph being assigned a score between 1 and 5 based on the extent of staining. A score of 1 represented negligible staining and a score of 5 represented intense, widespread staining.

4.3 Results

4.3.1 Ovarian morphology

No gross differences in the morphology of fetal ovaries attributable to maternal restricted nutrition were evident at both days 55 and 75. At day 55 of gestation, development of ovigerous cords was well advanced. Within the ovigerous cords, germ cell proliferation was noted by the presence of mitotic figures. Some germ cells showed the lack of a discrete nucleus but the presence of thread like chromatin material, illustrating that meiosis had been initiated in these cells (Figure 4.3 C and D). The presence of small eosinophillic germ cells with darkly staining condensed nuclei indicated that apoptotic germ cells were also present (Figure 4.4 A and B).

At day 75, the frequency of apoptotic bodies and meiotic germ cells was more prevalent than observed at day 55. Additionally, a small number of follicles were observed in all day 75 ovaries (Figure 4.3 E and F). A typical developmental gradient of germ cells was present with the most advanced germ cells (follicles) being evident in the inner most regions of the cortex (Figure 4.3 E and F), while the least developmentally advanced (oogonia) were predominantly located towards the periphery of the cortex (Figure 4.3 D).

Atresia of germ cells displayed a range of morphologies in both restricted and maintenance groups. Some germ cells displayed small dark condensed nuclei, often, although not always, accompanied by an eosinophilic cytoplasm. Other germ cells showed nuclei that had disintegrated, these cells in general did not show eosinophilia. A further morphology associated with germ cell atresia showed the presence of extremely large cytoplasmic vacuoles, these cells were often, but not always, associated with condensed nuclei. Some cells displaying atretic morphology did not stain for TUNEL, while others, also displaying atretic morphology, did not stain for MAPLC3. A range of germ cell morphologies associated atresia are presented in Figure 4.4.

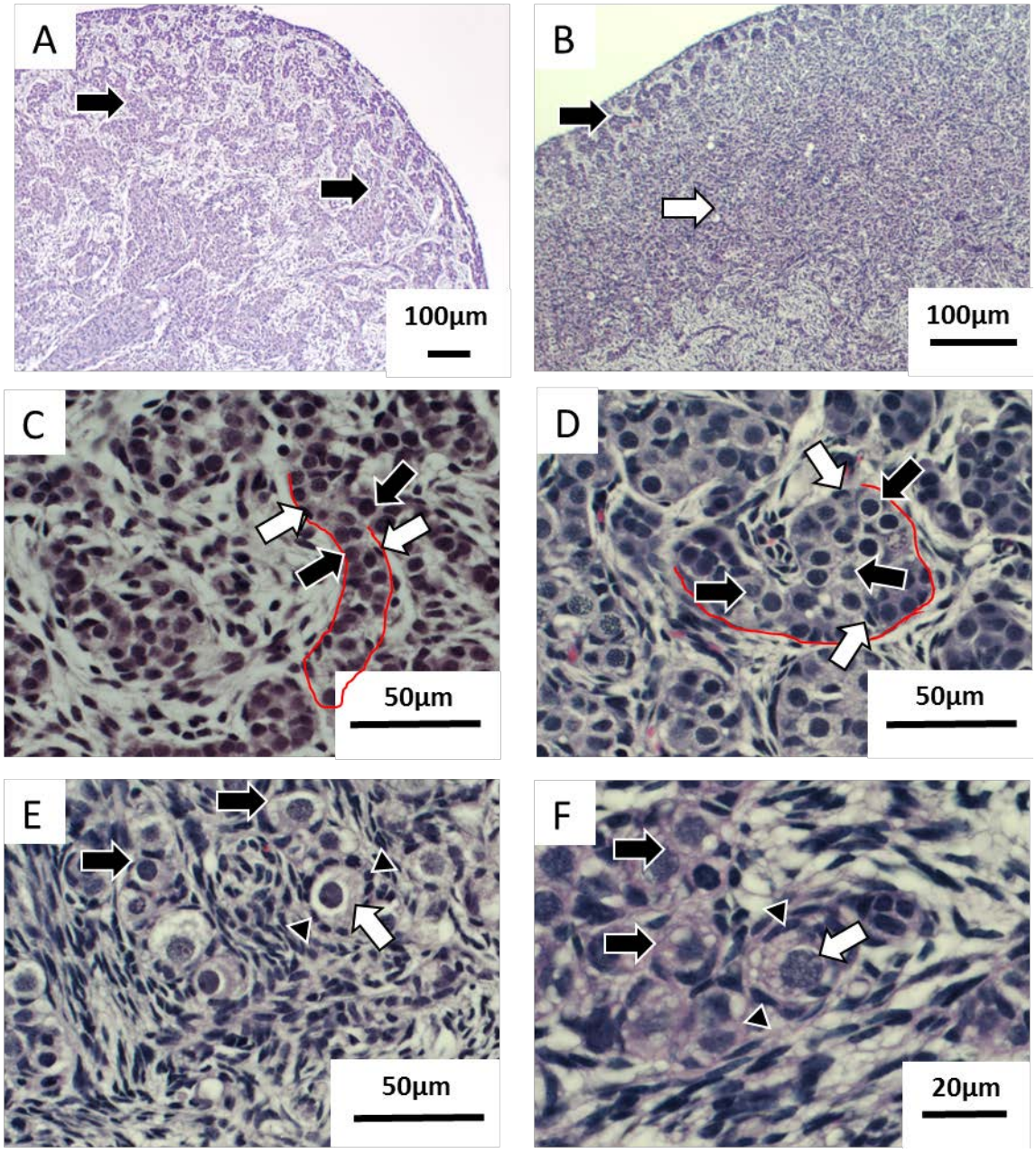

Figure 4.3 Ovarian morphology in H&E stained sections. (M) = maintenance ovary, (R) = restricted ovary. **(A)** Day 55 (M) and **(B)** Day 75 (M). Showing presence of ovigerous cords (black arrows). In **(B)** expansion of the cords in the innermost regions of the cortex makes delineation of individual cords difficult at this magnification (white arrows). **(C)** Day 55 (R) and **(D)** Day 75 (M). Showing well defined cords partially outlined in red. Black arrows indicate germ cells showing a variety of nuclear morphologies representing differing stages of meiosis/mitosis. White arrows indicate pre-granulosa cells. **(E)** Day 75 (R) and **(F)** Day 75 (M). Showing examples of forming follicles (black arrows) and formed, isolated follicles (white arrows) surrounded by granulosa cells (black arrowheads).

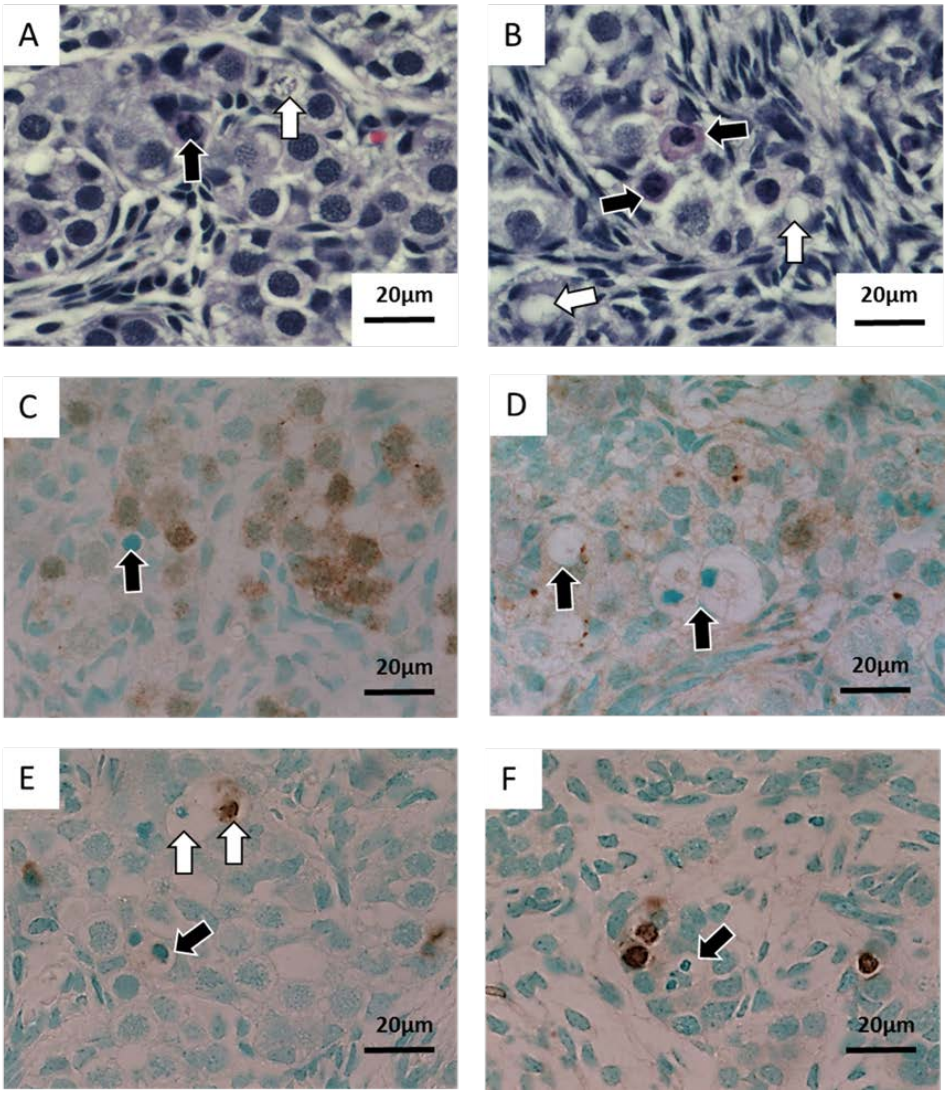

Figure 4.4 Morphologies associated with germ cell atresia. (M) = maintenance ovary, (R) = restricted ovary. **(A)** Day 75 (M). Germ cell with condensed heterogeneous nuclei and eosinophilic cytoplasm (black arrow) and germ cell with pale heterogeneous nuclei and pale cytoplasm (white arrow). **(B)** Day 75 (R). Germ cells with condensed nuclei and eosinophilic cytoplasm (black arrows) and germ cells with extremely large cytoplasmic vacuoles (white arrows). **(C)** Day 75 (M). MAPLC3 staining. Germ cell with condensed nuclei showing no staining for MAPLC3 (black arrow). **(D)** Day 75 (R). MAPLC3 staining. Germ cells with large cytoplasmic vacuoles showing no staining for MAPLC3 (black arrows). **(E)** Day 75 (M) TUNEL. Adjacent germ cells with large cytoplasmic vacuoles (white arrows), left hand cell showing no staining, while right hand cell shows some staining. Black arrow shows germ cell with disintegrated nuclei showing no staining for TUNEL. **(F)** Day 55 (R) TUNEL. Black arrow shows germ cell with disintegrated nuclei showing no staining for TUNEL.

4.3.2 Ovarian volumes and germ cell numbers

To test the accuracy of the sampling strategy used for stereology, two ovaries at each age were counted using twice the number of sections i.e. volumes on every 10^{th} section, and cell counts on every 25^{th} section. Results were then calculated for every 10^{th} and 25^{th} section and compared to results for the same ovaries calculated from every 20^{th} and 50^{th} section. For each of the two ovaries, the results did not differ by more than 10%, indicating the sampling strategy of every 20^{th} section for volumes and every $50^{th}/51^{st}$ section for cell counts was sufficient to produce reliable estimates. To ensure the consistency of the methodology, once all ovaries had been counted, one ovary at each age was recounted, with the duplicate values not differing from the original counts by more than 10%.

The pattern of germ cell development in maintenance ovaries is similar to that reported by Smith and colleagues (*38*). From day 55 to day 75, total germ cell numbers increased 2.4 fold, with numbers of oogonia remaining static and oocyte numbers increasing 11 fold, see Table 4.1.

The effects of gestational nutrition on germ cell numbers and ovarian volumes are presented in Table 4.1. Significant effects of gestational age ($p < 0.01$, GLM) and group ($p < 0.05$, GLM) were noted for ovarian volume with no significant gestational age x group interaction. Similarly for cortex volume, effects of gestational age ($p < 0.01$, GLM) and group ($p < 0.05$, GLM) were noted with no gestational age x group interaction. There were no significant differences between nutritional groups at day 55 of gestation for ovarian or cortex volumes (ANOVA). At day 75, ovaries from fetuses exposed to restricted nutrition were significantly larger ($p < 0.05$, ANOVA), with a larger cortex volume ($p < 0.05$, ANOVA) when compared to ovaries from fetuses exposed to maintenance nutrition.

Germ cell numbers, showed significant effects of gestational age ($p < 0.01$, GLM), and group ($p < 0.01$, GLM) with a significant gestational age x group interaction ($p = 0.02$, GLM). At day 55 of gestation, there was no significant difference between nutritional groups in germ cell numbers (ANOVA), an observation also reflected in similar numbers of both oogonia and oocytes between the groups. At day 75 of gestation, ovaries from fetuses exposed to restricted nutrition contained 46% more germ cells when compared to ovaries exposed to maintenance levels of nutrition ($p < 0.01$, ANOVA). When examined further, the increase in germ cell number in day 75 restricted fetal ovaries was reflected in both significantly more oogonia ($p < 0.05$, ANOVA) and more oocytes ($p < 0.05$, ANOVA). Germ cells classified as oocytes also

included the relatively small number of germ cells which were enclosed in isolated follicles. The number of follicles present was insufficient for accurate quantification.

Table 4.1 Germ cell numbers and ovarian volumes estimated by stereology

Volume or Cell number	Age	Maintenance	Restricted
Number of ovaries	Day 55	6	7
	Day 75	8	7
Ovary volume (mm^3)	Day 55	1.70 ± 0.19	1.93 ± 0.19
	Day 75	5.23 ± 0.37	6.39 ± 0.30*
Cortex volume (mm^3)	Day 55	1.34 ± 0.13	1.53 ± 0.14
	Day 75	3.81 ± 0.26	4.74 ± 0.24*
Germ Cell number	Day 55	317,461 ± 37,367	333,484 ± 42,472
	Day 75	766,344 ± 19,070	1,122,285 ± 87,235**
Oogonia	Day 55	274,217 ± 31,414	256,789 ± 28,677
	Day 75	293,499 ± 32,810	453,904 ± 32,810*
Oocytes	Day 55	43,243 ± 6,486	76,696 ± 21,649
	Day 75	472,845 ± 41,190	668,381 ± 71536*

Data are means and standard errors. * indicates $p < 0.05$, ** indicates $p < 0.01$ for between nutritional group comparisons.

4.3.3 Germ cell proliferation

Sections from the 28 fetal ovaries used for germ cell counts were used for all IHC protocols. Negligible staining was observed in both the negative controls (Section 4.2.4) (Figure 4.5 A) indicating that staining using the Ki-67 antibody was specific. Positive control sections of adult ovarian tissue showed extensive staining in follicular granulosa cells (Figure 4.5 B). Positive staining for proliferating cells was predominantly confined to germ cells, particularly those located towards the outer most regions of the ovarian cortex (Figure 4.5 C and D). Positively stained germ cells often occurred in groups or clusters (Figure 4, 5 C, E and F). Occasional cells of the ovarian surface showed positive staining (Figure 4.5 C and D). Pre-granulosa cells within the cords showed no staining (Figure 4.5 E and F). Considerable variation was noted in the number of stained cells between sections. Despite this variation, overall mean results were similar between most groups and ages. The densities of Ki-67 positive germ cells in the ovarian cortex are shown in Table 4.2.

Overall there was no effect of gestational age ($p = 0.6$, GLM) or group ($p = 0.06$, GLM) on the density of Ki-67 positive staining cells, with no significant gestational age x group interaction ($p = 0.2$). ANOVA reported no significant differences between nutritional groups at day 55, however at day 75, the density of proliferating germ cells in ovaries from restricted fetuses was one quarter of that observed in maintenance animals ($p < 0.01$, ANOVA). Comparative densities between ages and groups are illustrated in Figure 4.6.

Table 4.2 Density of proliferating (Ki-67 positive) germ cells

Age	Maintenance	Restricted
55	0.87 ± 0.43	0.76 ± 0.17
75	1.01 ± 0.32	0.26 ± 0.09**

Values means and standard errors of the number of positive staining cells per unit area of ovary. ** indicates $p < 0.01$ between group comparison.

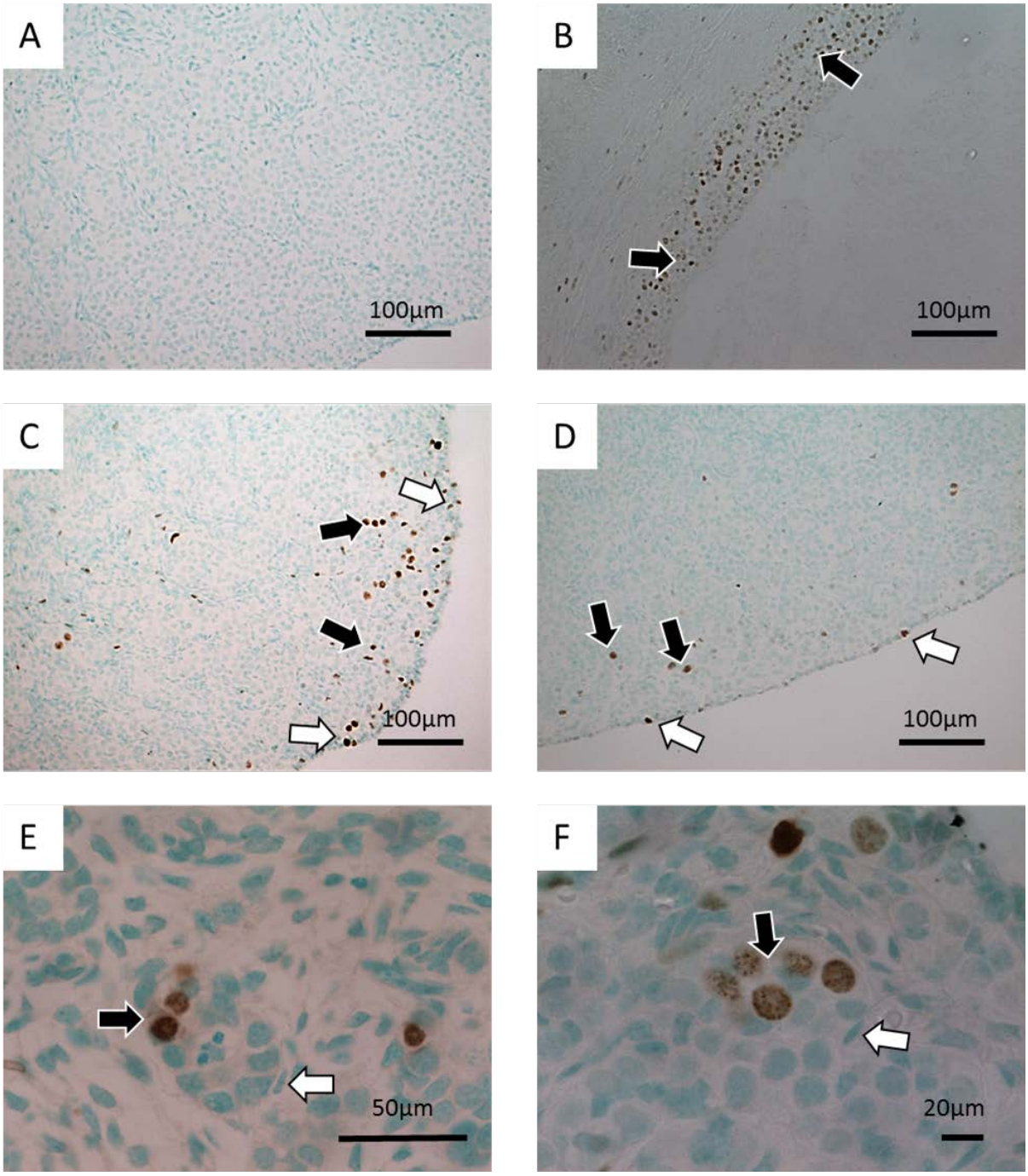

Figure 4.5 Staining for proliferating cells (Ki-67). (M) = maintenance ovary, (R) = restricted ovary. **(A)** Negative control. Incubated with non-immune serum showing no staining. **(B)** Positive control. Antral follicle from mature ovary showing extensive staining in granulosa cells. **(C)** Day 55 (M) and **(D)** Day 75 (R). Showing germ cells staining in the outer regions of the ovarian cortex (black arrows) with occasional staining in the ovarian surface (white arrows). **(E)** Day 55 (R) and **(F)** Day 75 (M). Showing staining specific to germ cells, often clustered (black arrows), while pre-granulosa cells remain unstained (white arrows).

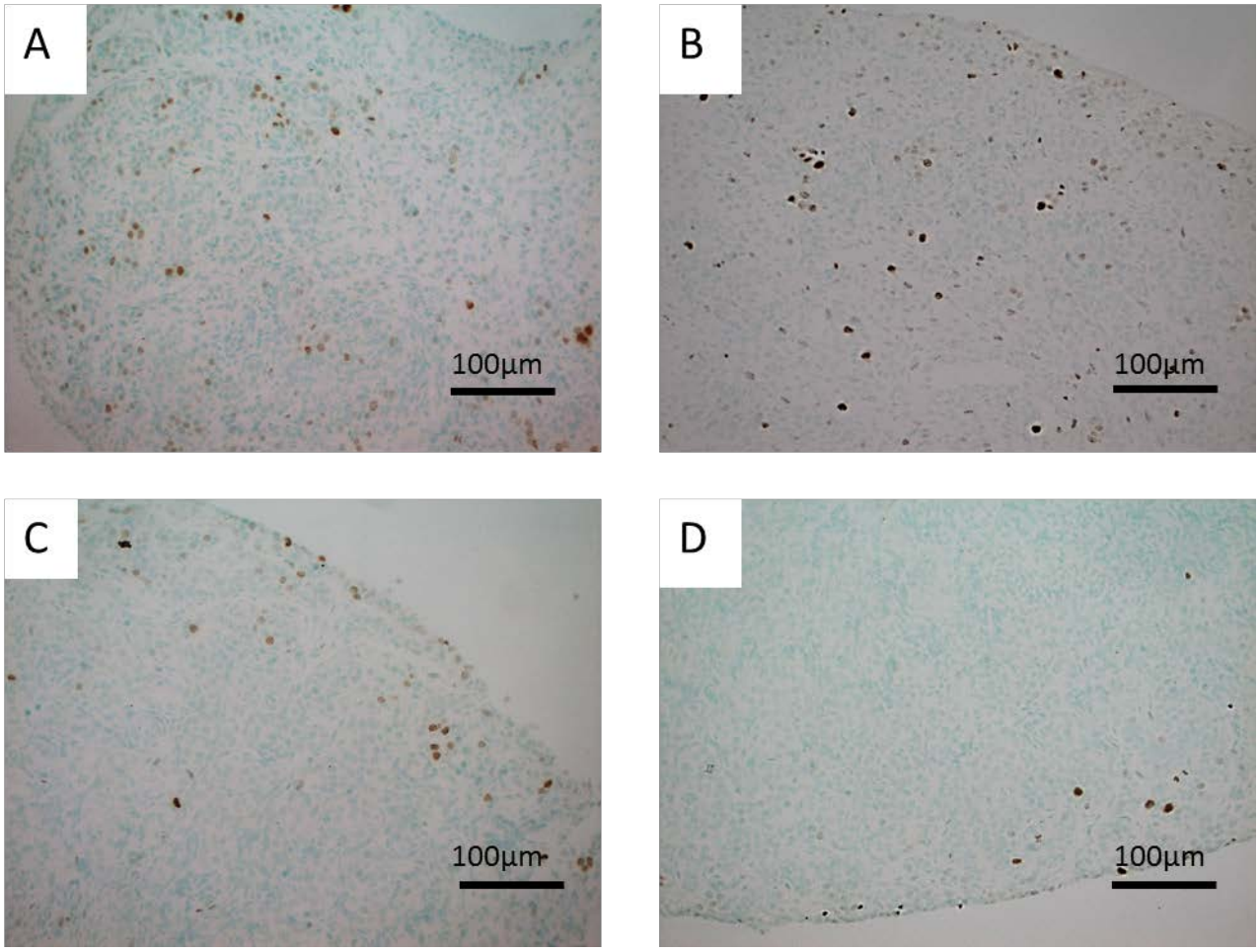

Figure 4.6 Comparative densities of proliferating cells. **(A)** Day 55 maintenance proliferating cell density approximately 0.9. **(B)** Day 55 restricted proliferating cell density approximately 0.9 **(C)** Day 75 maintenance proliferating cell density approximately 0.85. **(D)** Day 75 restricted proliferating cell density approximately 0.2.

4.3.4 Autophagy

Staining with the negative control antibody was negligible (Figure 4.7, A), indicating that the staining observed was specific for MAPLC3. At day 55, staining for MAPLC3 was predominantly confined to the ovigerous cords throughout the ovarian cortex (Figure 4.7 C and E). Within the ovigerous cords, staining appeared to be exclusively within the germ cells, with pre-granulosa cells showing no staining (Figure 4.7 B). At day 75, staining was significantly less intense ($p < 0.05$, ANOVA) and concentrated in the outer regions of the ovarian cortex (Figure 4.7 D and F). In general, germ cells that had completed meiosis displayed less intense staining than those actively undergoing meiosis, while those germ cells within forming or isolated follicles displayed little or no staining.

With regards to assessment and grading of micrographs, the three independent graders assigned identical scores to 46% of the micrographs. For the remaining 54% of the micrographs, two graders assigned identical scores, while one grader's score differed by 1 (e.g. the three scores assigned were 3, 3, 4). There was no individual grader who consistently differed in scores from the others. While it is accepted that the method applied is subjective, the consistency between graders gives a high level of confidence to the mean scores. In Figure 4.7, plates C and E are indicative of a score of 5, plate D a score of 3 and plate F a score of 2. The relative staining intensities are presented in Table 4.3.

Table 4.3 Relative staining for autophagy (MAPLC3)

Age	Maintenance	Restricted
55	3.1 ± 0.3	2.9 ± 0.2
75	2.2 ± 0.4	2.5 ± 0.3

Relative staining was scored between 1 (light sparse staining), and 5 (intense widespread staining). No effect of group was noted.

A significant effect of gestational age on MAPLC3 staining was noted ($p < 0.05$, GLM) with less staining apparent at day 75 when compared to day 55. No overall effect of group was noted, and no gestational age x group interaction was noted (GLM). No group differences in the relative staining for MAPLC3 were observed between nutritional groups at either day 55 or day 75 of gestation by ANOVA.

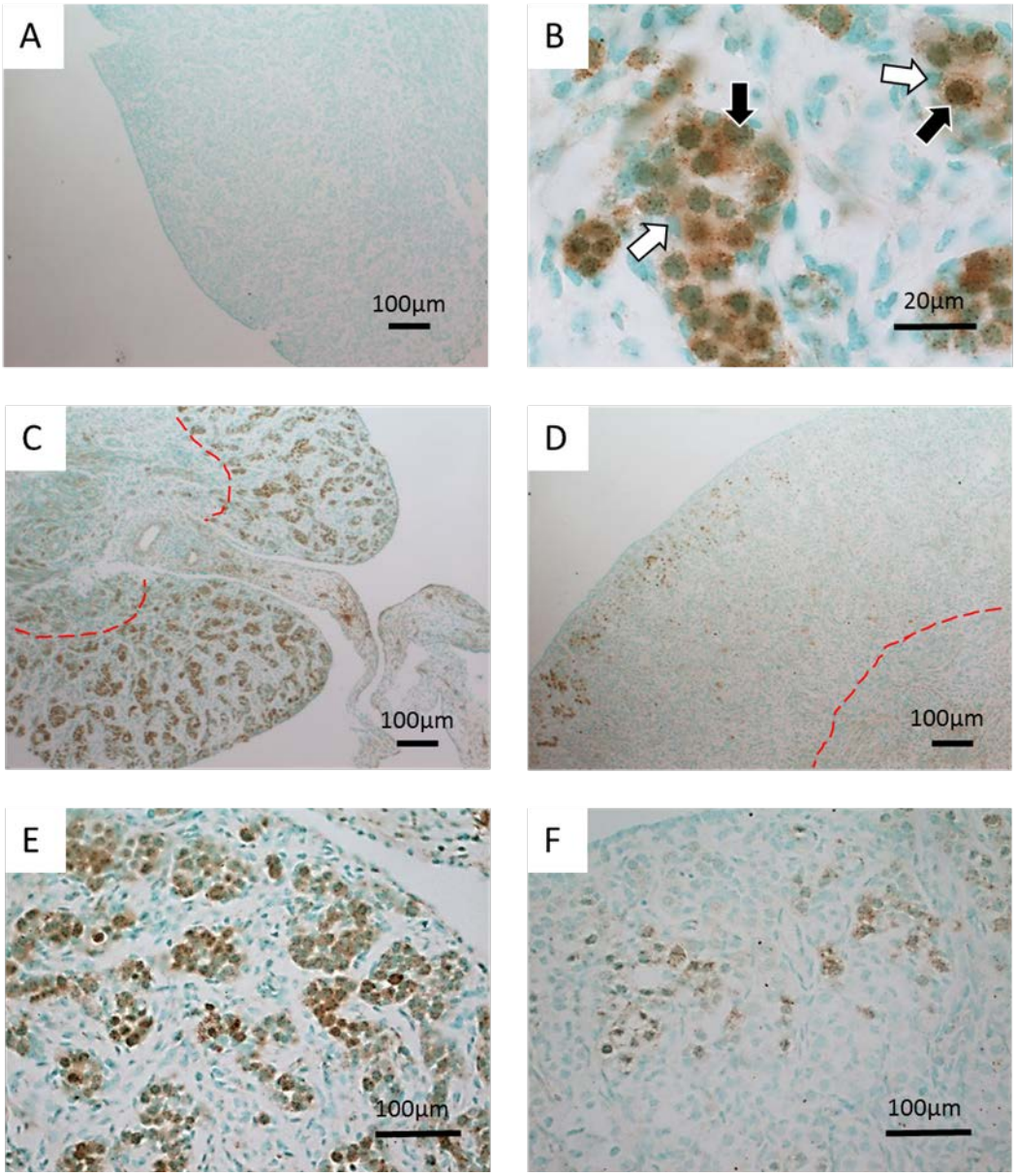

Figure 4.7 Staining for autophagy (MAPLC3). (M) = maintenance ovary, (R) = restricted ovary. **(A)** Negative control. Incubated with non-immune serum showing no staining. **(B)** Day 55 (M). Showing staining confined to germ cells (black arrows) while pre-granulosa cells show no staining (white arrows). **(C)** Day 55 (M) and **(D)** Day 75 (M). Showing reduced cortical staining at day 75 compared to day 55, the cortical–medullary boundary indicated by dashed line. **(E)** Day 55 (R) and **(F)** Day 75 (R). Showing both reduced staining area and intensity at day 75 compared with day 55 which shows positive staining largely confined to ovigerous cords throughout the ovarian cortex.

4.3.5 Germ cell apoptosis

Negligible staining was apparent on negative control slides (removal of the TdT enzyme) (Figure 4.8 A), while intense staining over the entire section was observed on positive control slides (addition of the nuclease enzyme, Section 4.2.4) (Figure 4.8 B). This indicates the protocol is specifically staining breaks in DNA. The pattern of TUNEL staining at both days 55 and 75 indicated that the majority of apoptotic cells were germ cells located in the ovigerous cords of the ovarian cortex (Figure 4.8 E and F). The few exceptions to this observation were occasional vascular endothelial cells and cells of the connecting rete in the ovarian medulla. There appeared to be no discernible pattern related to the distribution of apoptotic germ cells. Positive cells occurred throughout the ovarian cortex both individually (Figure 4.8 C) and occasionally in clusters (Figure 4.8 D). The relative densities of apoptotic germ cells are presented in Table 4.4.

No effect of gestational age was noted on the density of apoptotic cells, ($p = 0.07$, GLM). There was no overall effect of group ($p = 0.5$) and no gestational age x group interaction. There were no significant differences in the number of apoptotic cells attributable to nutritional group at either day 55 or day 75 of gestation (ANOVA).

Table 4.4 Number of apoptotic cells per unit area of ovary

Age	Maintenance	Restricted
55	2.0 ± 0.5	1.3 ± 0.2
75	1.6 ± 0.3	1.2 ± 0.1

Values are means and standard errors.

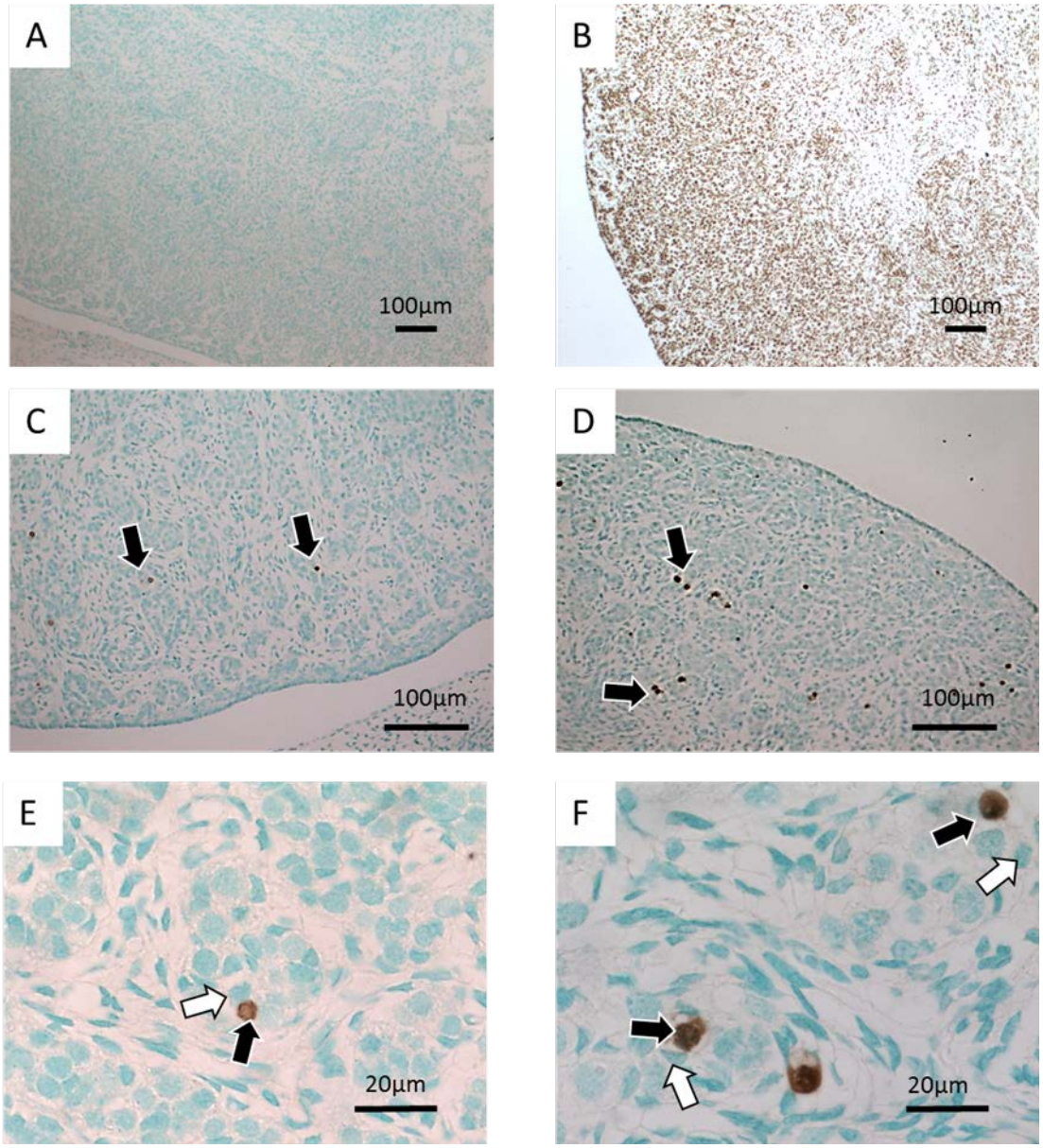

Figure 4.8 Staining for apoptosis (TUNEL). (M) = maintenance ovary, (R) = restricted ovary. **(A)** Day 75 (M). Negative control. Without TdT enzyme showing no staining. **(B)** Day 75 (R). Positive control. With addition of nuclease enzyme. **(C)** Day 55 (M), and **(D)** Day 75 (R). Showing variation and random appearance of apoptotic germ cells (black arrows) throughout the cortex. **(E)** Day 55 (R), and **(F)** Day 75 (M). Ovigerous cords with positive staining apoptotic germ cells (black arrows). Pre-granulosa cells within the cord do not stain (white arrows).

4.4 Discussion

Stereology and IHC were used to analyse fetal ovaries exposed to either maintenance or restricted nutrition to determine whether the nutritional restriction affected germ cell and ovarian development.

The absence of significant group effects at the cessation of nutrition restriction (day 55), is consistent with the concept that restricted gestational nutrition, at levels used in this study, had no direct effect on fetal ovarian or germ cell development as assessed by these morphological criteria. The differences in maternal metabolic factors and endocrinology at day 55 of gestation reported earlier (Section 3.3.2) do not appear to have directly affected germ cell proliferation, apoptosis, or autophagy as evidenced by staining for markers of these processes (Ki-67 for proliferation, TUNEL for apoptosis and MAPLC3 for autophagy). It is however, intriguing that 20 days following cessation of restricted maternal nutrition, germ cell numbers in ovaries from fetuses exposed to restricted nutrition were significantly increased when compared to fetal ovaries exposed to the maintenance diet. Thus, the observed changes to germ cell numbers may not be the result of restricted nutrition, but the result of the change from restricted nutrition to ad libitum nutrition. This does not preclude the possibility that the restricted nutrition produces delayed effects on ovarian development. Such a scenario has been demonstrated in sheep by Rae and colleagues (*178*) where gestational undernutrition for the first 30 days of pregnancy produced an effect on follicle development at day 100 of gestation. However, this period of nutrition (mating – day 30) did not affect ovarian development at day 65 of gestation. Thus the differences in germ cell numbers reported at day 75 in the current study appear less likely to be a delayed response to the earlier undernutrition. Of interest with respect to fetal exposure to endocrine disrupting chemicals (EDCs), Lea and colleagues (*284*) conclude that a change in fetal environment appears to be more detrimental than a consistent exposure to EDCs. While the current study focuses on differing levels of nutrition, the emerging hypothesis is similar to the conclusion of Lea and colleagues in that it is the change in fetal environment which may be responsible for the observed effects

The possibility of the change in nutritional status (from restricted to ad libitum and the growth spurt shown by restricted animals) being ultimately responsible for the effects seen in adult animals was raised during the discussion in Chapter 2. The germ cell data provides further evidence that this change in status may be the key in establishing the observed effects. Additionally, the data from Chapter 3 was consistent with the effects seen in adult ewes being

ovarian driven. (Section 3.4). The fetal ovary data, showing nutritionally induced changes to fetal germ cell development, support the hypothesis that the observed effects thus far are ovarian driven.

Studies to date have relied on germ cell densities (*159, 171*) when considering germ cell numbers, failing to account for ovarian volume and more importantly ovarian cortical volume. Germ cells are confined to the ovarian cortex and as the fetal ovary develops and germ cell numbers increase, the cortical volume increases to accommodate these additional germ cells (*38*). Following the substantial wave of atresia and the loss of approximately 80% of germ cells between days 75 and 90 of gestation, cortical volume decreases (*38*). This implies that changes in fetal ovarian germ cell numbers are unlikely to be reflected in the density of these cells, but in the volume of the ovarian cortex. This concept is supported by the observation in this study where the increased numbers of germ cells in restricted ovaries at day 75 of gestation is accompanied by a corresponding increase in cortical volume. Further, a 1.25 fold increase in cortex volume and a 1.5 fold increase in germ cell number implies that the density of germ cells between the two groups may be quite similar. In the current study, the 1.5 fold increase in germ cell numbers observed at day 75 translates to a 1.18 fold increase in germ cell density based on both ovarian volume and cortex volume, potentially making this smaller difference in density more difficult to detect. The use of stereological methods in this study overcomes the issues associated with cortical or ovarian volumes, and for the first time provides accurate, unbiased estimates of germ cell numbers in fetal ovaries exposed to restricted maternal nutrition.

One aspect of germ cell development not studied in detail in the current study is meiosis. Quantification of cells positive for factors such as *STRA8* or *DAZL*, expressed in meiotic or arrested germ cells, could establish whether restricted nutrition has affected levels or timing of meiosis. However, were alterations to the regulation of meiosis to be the mechanism underlying the increased germ cell numbers at day 75, then one might expect to see either increased oogonia at day 75 (reduced meiosis), or increased oocytes (increased meiosis) at day 75, but not both simultaneously. Restricted ovaries contain 1.5 fold more total germ cells, 1.5 fold more oogonia, and 1.4 fold more oocytes. The similarities in these fold changes would suggest that changes to meiosis is not the mechanism underlying increased numbers of germ cells. The mechanism, be it proliferation, apoptosis, survival, or some other mechanism, by which increased germ cell numbers arise in restricted ovaries, may be active in a brief transition period following the change in nutrition levels.

The finding of lower proliferation rates at day 75, when germ cell numbers are higher, is consistent with an advancement in the timing of germ cell development in ovaries from restricted fetuses, and that ultimately, germ cell numbers in maintenance ovaries (where proliferation rates are higher than restricted ovaries) may reach parity with germ cell numbers in restricted ovaries. The similar levels of apoptosis at day 75 would support this contention. Conversely, female offspring from the dams exposed to restricted gestational nutrition have a higher AFC than those from maintenance dams (Section 2.3.6), and as a relationship has been demonstrated between AFC and ovarian reserve, it would seem that the differences in germ cell numbers observed at day 75 may lead to differences in ovarian reserve. It remains to be seen whether this apparent alteration in germ cell development leads to differences in ovarian reserve, or whether germ cell numbers in maintenance ovaries reach similar levels, but at a later age. Future examination of fetal ovaries in late gestation would clarify any effects on ovarian reserve. Additionally, examination of fetal ovaries between day 55 and day 75 may identify the mechanism by which increased germ cell numbers are observed at day 75.

IHC results for Ki-67 showed wide variation in the numbers of proliferating germ cells between individual sections within an ovary. Further, staining often occurred in small clusters. These effects may be due to the location of positive cells with respect to structures such as developing blood vessels, or concentrations of stromal cells, both possible sources of factors regulating germ cell proliferation. The lower density of proliferating germ cells at day 75 is consistent with the study of Lea and colleagues who reported decreased levels of Ki-67 staining at day 65, following restricted maternal nutrition between days 31 to 50 (*171*).

In the same study, Lea and colleagues also suggested a link between restricted nutrition and increased expression levels of the pro-apoptotic factor *BAX* at day 65. This would suggest increased apoptosis and consequently fewer germ cells in restricted ovaries, an observation not supported by the data presented in this work. In the current study, levels of germ cell apoptosis were not different between the groups at either day 55 or day 75. Regulation of apoptosis is often considered a balance between a number of BCL-2 family members including the pro-apoptotic genes (e.g. *BAX, BAK, BOK, BLK, BNIP, BAD, BID, BIM and EGL-1*), and anti-apoptotic genes (e.g. *BCL2, BCL-XL, MCL-1, A1, BCL-W*) (*285*). Additionally the list of genes and factors that contribute to the regulation of apoptosis is considerable (*51*) with any number of these factors potentially being affected by restricted nutrition. Results of the gene expression analysis (Chapter 6), will offer more insights into the regulation of apoptosis in these fetal ovaries, particularly whether *BAX* is up-regulated as in the study of Lea and colleagues.

The similarities in the levels of apoptosis (as detected by TUNEL) between groups however, suggest that apoptosis has not been affected by the restricted nutrition at the ages examined. While apoptosis is considered the primary mechanism by which fetal germ cells are lost (*286*), there is the likelihood of alternative mechanisms of germ cell loss not examined in the current study. The range of morphologies shown in Figure 4.4 along with lack of staining for TUNEL and MAPLC3 for many germ cells showing evidence of cell death would seem to support this concept. Both Wartenburg and colleagues (*287*) and McClellan and colleagues (*288*) report a novel non-apoptotic cell death pathway which may be responsible for a considerable amount of germ cell loss. Given this, while levels of apoptosis and autophagy between the nutritional groups is similar at both ages, differences in germ cell loss between groups cannot yet be discounted as a mechanism underlying the observed difference in germ cell numbers.

The use of IHC to identify, and quantify autophagy is a novel approach to the assessment of experimentally induced differences in germ cell development. The staining pattern observed, with staining less intense and less widespread at day 75 when apoptosis of germ cells is increasing markedly, appears to fit the findings of Gawriluk and colleagues (*289*). In the mouse, Gawriluk and colleagues concluded that autophagy may be an important regulator of germ cell survival prior to the formation of the primordial follicle pool. In the rat however, Escobar and colleagues propose a role for autophagy in oocyte death (*290*) with the majority of dying oocytes simultaneously displaying features of apoptosis (TUNEL and caspase 3), and autophagy (increased acid phosphatase activity and IHC for LAMP1). The focus of the Escobar study appears to be on oocytes within follicles, whereas in the current study and that of Gawriluk, the focus is on pre-follicular oocytes. While some follicles have formed at day 75, staining for MAPLC3 in the germ cells in these follicles is not apparent. Further, the germ cells within the newly formed follicles do not appear to show apoptosis. This lack of staining in follicular oocytes may reflect the low numbers of follicles present at this age. However, it is in agreement with the findings of Fulton and colleagues who note low germ cell death following follicle formation, a result of a protective follicular environment (*48*). Both the pattern of MAPLC3 staining and expression levels of autophagy associated genes at later gestational ages may indicate whether a shift in the function of autophagy is apparent between pre-follicular oocytes and follicular oocytes. The relative expression levels of autophagy associated genes has been proposed as a method of determining the role (either cell survival or cell death) of autophagy in a given tissue. Expression levels of these genes will be examined in Chapter 5.

Overall, the results from the analysis of fetal ovaries in this section show no direct or immediate effect of nutritional restriction on germ cell development at day 55 as assessed by germ cell numbers, proliferation rates, apoptosis rates, and levels of autophagy. However, following cessation of nutritional restriction, an increase in germ cell numbers is observed in ovaries exposed to restricted nutrition compared to maintenance ovaries. The mechanism by which this increase occurs is unclear, and may be active for only a brief period following cessation of nutritional restriction. The emerging hypothesis is that rather than nutrition restriction, it is the change from restricted to ad libitum nutrition which has established the effects seen in this study.

Alterations to nutrition are known to have impacts on the gene expression patterns of a number of mammalian cell types (*291*). Restricted gestational nutrition or a change in nutrition (from restricted to ad libitum) may affect genes or pathways involved in germ cell development. Identification of alterations in gene expression in fetal ovaries may provide some insights into the mechanism(s) underlying the observed changes. Additionally, analysis of expression levels of autophagy associated genes may help determine the functional role of autophagy in the fetal ovary at this stage of development.

Chapter 5 . Application of RNAseq to Fetal Ovaries

5.1 Introduction

The data presented in the preceding chapters demonstrates that the nutritional restriction regime employed in this study increased indicators of fertility in adult female offspring, with these changes appearing to be ovarian in origin. The increased indicators of fertility observed in female offspring are contrary to most published studies in this field (*167, 173*). These changes may stem from differences in fetal ovarian development, given that this is when restricted nutrition was applied, and that differences in fetal germ cell numbers are apparent at day 75 (Section 4.3.2). How nutrition affects events at the cellular level is likely to hold the key to understanding the relationship between nutrition, ovarian development, and postnatal fertility. Identifying the mechanisms underlying this relationship may pave the way for the development of practical applications to improve fertility in domestic livestock.

RNAseq was considered the ideal method for the current study for several reasons. Firstly, RNAseq examines the whole transcriptome. Providing sample size and sequencing depth are adequate, data will be generated for all genes that express mRNA (*292*). Secondly, while not a focus of this study, RNAseq will provide expression data for gene splice variants.

The data from the current study and previous studies suggest alterations to germ cell development may underlie the effects of gestational nutrition (*10, 159*). This study uses whole fetal ovaries to examine gene expression. Isolation and characterisation of germ cells is feasible for a study of this nature, however such a strategy would ignore the potential roles of other ovarian cell types. Additionally, the acute cell stress resulting from any isolation process is likely to alter gene expression from the native state.

At day 55 of gestation, pre-granulosa cells are in close contact with germ cells (*34*). It is thought that additional pre-granulosa cells are being recruited from cells near the ovarian surface (*28, 29*), with these cells most likely derived from GREL cells (*28*). In the rodent it has been shown that the mesonephros or mesonephros-derived cells are critical for producing the signal(s) initiating germ cell meiosis, primarily retinoic acid (*44*). At day 55 in the sheep fetal ovary, migrating mesonephric cells form a large distinct structure in the ovarian medulla. Both pre-granulosa and mesonephric derived cells seem likely to play a significant role in germ cell

development. Thus, the strategy of using whole fetal ovaries will allow the possible roles of these cell types to be considered.

While the focus of this study is on differential gene expression between fetal ovaries from maintenance and restricted dams (presented in Chapter 6), analysis of expression of autophagy related genes may help to determine whether autophagy is involved in cell survival or cell death in sheep fetal ovaries at the ages examined in the current study (day 55 and day 75). The same proteins/genes are involved in both the cell death and cell survival aspects of autophagy. However, Tsujimoto and Shimizu (*293*) concluded that the level of expression of the autophagy associated genes *ATG5* and *ATG6* can distinguish between the cell death and cell survival processes. During the cell survival process, the expression levels of *ATG5* and *ATG6* remain low, but are highly up-regulated during autophagic cell death when compared to other autophagy related genes (*293*). While the evidence from Section 4.3.4 suggests that autophagy is playing a role in cell survival, expression levels of autophagy related genes may provide further supporting evidence for this contention.

Therefore, the aim of the work presented in this chapter was to use RNAseq to generate high quality data describing the expression of genes in day 55 and 75 fetal ovaries. This data will be related to developmental processes previously discussed in this thesis. Analysis of the data generated for differential gene expression will be presented in Chapter 6.

5.2 Materials and methods

5.2.1 The concept of RNAseq

The fundamental concept of RNAseq involves the alignment of short, sequenced fragments of cDNA generated from sample mRNA to a reference genomic database (*292*). A reference genomic database is a complete nucleic acid sequence of DNA from a particular species. Over time these databases become increasingly annotated (where particular sequences within the database are assigned to specific genes). Therefore, sample sequences aligned to database sequences can be assigned to specific genes.

The process from a fetal ovary to gene expression data is complex and has been illustrated in Figure 5.1. More than one technical option is available to achieve the desired outcome at most steps in the process. In Figure 5.1, the steps highlighted in bold font indicate where more than one option was trialled in this study.

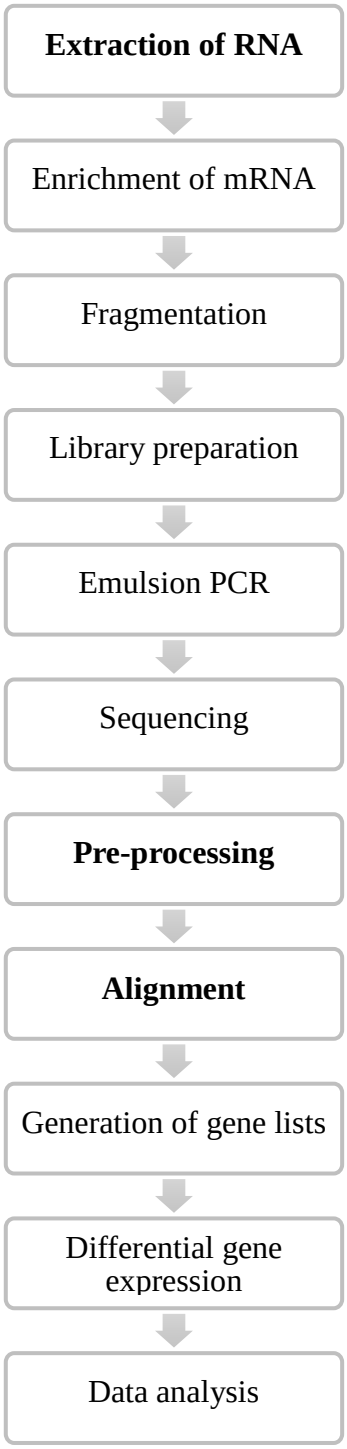

Figure 5.1 Diagram outlining workflow from RNA extraction to data analysis. Items in bold indicate steps where more than one protocol was trialled. Differential gene expression and data analysis are presented in Chapter 6.

5.2.2 Extraction of total RNA

5.2.2.1 Assessment of RNA extraction procedures

Selection of a nucleic acid extraction procedure considered isolation of both DNA and RNA from a single sample. DNA isolation was included for prospective future epigenetic studies. Fetal testes were used to compare the performance of two technologies. These technologies were: Trizol-chloroform based extraction procedures (easy-BLUE, INtRON Biotechnology, South Korea), and column based extraction procedures (Zymo Duet, Zymo Research, Irving, CA, USA).

For these trials, RNA concentrations, fragment size distributions, and quality were measured using a Bioanalyser (Agilent 2100, Agilent Technologies, Santa Clara, CA, USA). The RNA 6000 pico kit (Agilent Technologies) was used following the manufacturer's instructions with samples diluted 1:200. The Bioanalyser uses miniaturised versions of agarose gels (electropherograms) within multi-well chips. Chips and kits are specific for the nucleic acid being measured and its expected concentration. RNA quality is reported as a referential integrity value (RIN). Figure 5.2 compares electropherogram traces from samples with high quality and low quality RNA. Additionally, Figure 5.2 gives an overview of how RIN values are calculated.

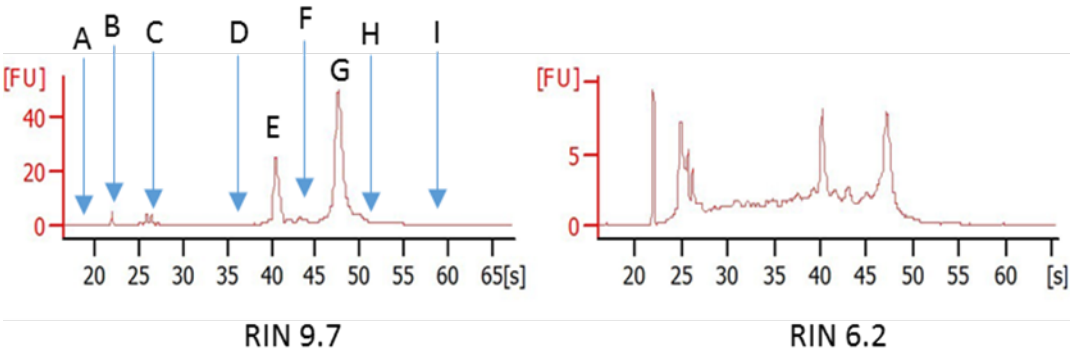

Figure 5.2 Calculation of RIN values for RNA quality assessment. Electropherogram traces showing profiles for high quality RNA (RIN 9.7), and low quality RNA (RIN 6.2). In calculating a RIN value, algorithms compare the area, intensities and ratios of the indicated areas: A = pro region, B = marker, C = 5S region, D = fast region, E = 18S fragment, F = inter region, G = 28S fragment, H = precursor region, and I = post region.

RIN values range from 1 (very poor quality) to 10 (very high quality). Reported lower limits of RIN values used in RNAseq vary, from as low as 3.9 to as high as 8. However, decreasing RNA quality can affect the expression results obtained (*294*), thus a RIN value of 7 is widely considered the minimum required for RNAseq. DNA contamination of RNA samples was measured using a Qubit assay (dsDNA BR, Thermo Fisher Scientific, Auckland, NZ) following the manufacturer's instructions.

5.2.2.2 RNA extraction using Trizol

Trizol procedures are based on organic extraction resulting in the separation of RNA into an aqueous phase, proteins and lipids into an organic phase, and DNA into an interphase between the aqueous and organic phases (*295*, *296*).

Trizol extraction largely followed the manufacturer's guidelines. Testes were homogenised (Ultraturrex, Ika Labotechnik T25) in 600 µL of Trizol (based on 1 mL per 50 to 100 mg of tissue). Phase separation was achieved by the addition of 200 µL of chloroform and incubating at RT for 3 minutes. Following centrifugation at 13,000 g for 10 minutes at 4°C (Heraeus Fresco 17, Thermo Fisher Scientific, Auckland, NZ), the upper aqueous layer containing RNA was collected. RNA was precipitated by the addition of 400 µL of isopropanol, incubating for 10 minutes at RT and centrifuged (12,000 g for 5 minutes at 4°C). The pellet was washed using 1 mL of 75% ethanol, and centrifuged (12,000 g for 5 minutes at 4°C). Following aspiration of the ethanol, the pellet was air dried at RT for 5 minutes and dissolved in nuclease free water.

The narrow interphase layer (between the upper aqueous phase and the lower phenol phase) contained DNA. This layer was also collected and the DNA precipitated by addition of 200 µL of ethanol and centrifugation at 2000 g for 5 minutes at 4°C. Two washing steps were performed by re-suspending in 0.1M sodium citrate in 10% ethanol, incubating for 30 minutes at RT and centrifuging (2000 g for 5 minutes at 4°C). The pellet was resuspended in 1.5 mL of 75% ethanol and incubated for 15 minutes at RT. Following a final centrifugation at 2000 g for 5 minutes at RT, the ethanol was aspirated and the pellet air dried for 5 minutes at RT. The DNA pellet was dissolved in 8 mM NaOH.

Variations to the manufacturer's protocol tested included alterations to reagent volumes to increase DNA recoveries or concentrations. Differing ratios of Trizol:chloroform:ethanol used were: 600:200:200, 600:400:400, 600:100:100, 600:200:400, and 600:400:600. Secondly, the use of an agarose based phase lock gel (PLG Heavy, 5 Prime, Gaithersburg, MD, USA) was tested. This gel creates a physical barrier between the aqueous and interphase layers, improving

purity of both the RNA and DNA recovered. Following the manufacturer's protocol, 600 µL of phase lock gel was added to the Trizol:chloroform mix. Following centrifugation, the phase lock gel formed a barrier between the aqueous and organic phases facilitating the removal of each layer. Finally, following the completion of the extraction procedure, purification of RNA using column technology was trialled (RNeasy Mini Kit, Qiagen, Hilden, Germany, or Zymo spin IIIC columns, Zymo Research Corporation, Irving, CA, USA). The manufacturer's protocols were followed for these post extraction procedures. Trizol protocols and results are presented in Table 5.1 (Section 5.3.2).

5.2.2.3 RNA extraction using binding columns

Column based procedures utilise the properties of nucleic acids to bind to silica filters under specific buffer conditions (*297*).

Column extraction used the Zymo Duet kit (Zymo Research Corporation, Irving, CA, USA. Cat D7001) initially following the manufacturer's protocol. In-column DNase digests were performed using the supplied reagents following the manufacturer's guidelines. Additional DNase digests were performed using the Ambion DNA-free kit following the manufacturer's guidelines (Ambion, Carlsbad, CA, USA. Cat 1906). Homogenised samples were first centrifuged (13,000g for 1 minute at RT, Eppendorf Minispin. Eppendorf, South Pacific, Ryde, NSW, Australia) through a DNA binding column with the flow-through then centrifuged through an RNA binding column. Nucleic acids were eluted from their respective columns and assessed. This kit is designed for use with cell suspensions, and initial results with fetal testes led to the columns quickly reaching their maximum binding capacity resulting in low RNA and DNA yields, as well as high DNA contamination of the RNA sample. Variations to the manufacturer's protocol were aimed at overcoming this issue, and are described alongside the results in Table 5.2 (Section 5.3.2). The most efficient modified protocol is outlined in Figure 5.3, and is also shown in Table 5.2 as Protocol O (Section 5.3.2).

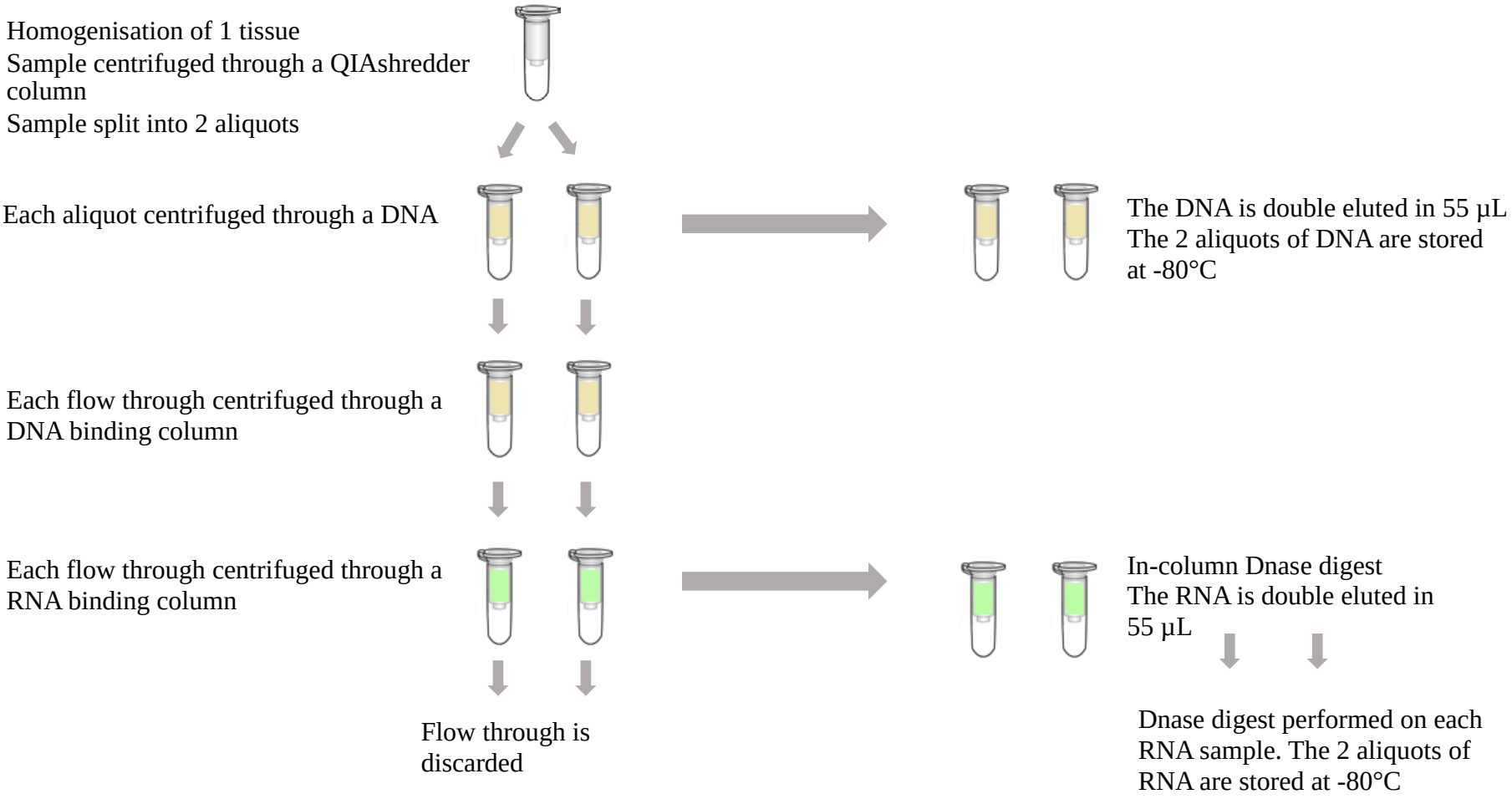

Figure 5.3 Optimised protocol used for RNA extractions.

5.2.3 Confirmation of fetal sex by PCR

Sex determination of sheep fetuses at day 55 and day 75 is a straight forward procedure based on the appearance of external genitalia (i.e. the presence of absence of a scrotum). Additionally, fetal testes are twice the weight of fetal ovaries at these ages. However, as a further safeguard to ensure identification and labelling procedures were correctly implemented, DNA extracted from fetal ovaries (Section 5.2.2) was used for PCR based sex determination. Primers were designed against ovine SRY and produce a 527 base pairs (bp) PCR product and ovine β-actin to produce a 420 bp PCR product (See Appendix E). Primers were sourced from Integrated DNA Technologies (IDT Pte. Ltd. Baullkham Hills, NSW, Australia). Samples showing the presence of both SRY and β-actin were designated male, while samples showing the absence of SRY but the presence of β-actin were confirmed as female. The method has been previously described by Lun (*33*). Briefly, a cocktail was prepared of 12.5 µL PCR master mix (Platinum^{TM} Hot Start Master Mix, Thermo Fisher, Auckland, NZ. Cat 13000013), 1 µL of forward primer, 1 µL of reverse primer (10 pmol/µL), and 10 µL of DNA sample (20 to 50 ng/µL). Following the PCR reaction (Eppendorf Mastercycler. Eppendorf South Pacific, Ryde, NSW, Australia), electrophoresis of the PCR products was performed on a 2% agarose gel containing ethidium bromide (10 µL of 10 mg/mL of ethidium bromide per 100 mL of agarose). Product sizes were determined by comparison to a standard size marker (1kb Plus, Thermo Fisher Scientific, Auckland, NZ, Cat 10787018). Electrophoresis gels were visualised on a Bio Rad Gel documentation system using Quantity One software (V4.6.3) (Bio Rad Scientific Ltd, Hercules, CA, USA). SRY product was sequenced by the University of Waikato sequencing facility (WDSF, University of Waikato, Hamilton, NZ). Using the National Centre for Biotechnology Information (NCBI) nucleotide blast tool, the sequence was compared to the NCBI nucleotide collection. For primer sequences and PCR cycling conditions see Appendix E.

5.2.4 ERCC spike-in control mixes

ERCC (External RNA Control Consortium) control mixes are synthetic RNA fragments of known sequence, sizes, and concentrations. Two spike-in mixes were used, each mix containing 92 polyadenylated transcripts. The transcripts within each mix span a wide range of concentrations. Subsequent analysis of the known concentration of these controls compared to the measured concentration allows an assessment of the dynamic range of the data, (i.e. the lower limit of detection and upper limit of detection).

Each of the 2 mixes contain the same transcripts at defined concentration ratios of 0.5, 0.67, 1, and 4 relative to each other. Comparison of the known concentration ratios to the measured

concentration ratios allows assessment of the fold change data generated from experimental data.

ERCC control mixes (Ambion ERCC Spike-In Control Mixes, Cat 4456739. Thermo Fisher Scientific, Auckland, NZ)) were added to total RNA samples (6 µL of the supplied reagent at a 1:100 dilution in nuclease free water) prior to mRNA enrichment (Section 5.2.5) with 12 samples receiving ERCC mix 1, and 11 samples receiving ERCC mix 2. Concentration and fold change values for the ERCC control mixes are given in Appendix F. Analysis was based on user guide published by the manufacturer (*298*). Data for each ERCC transcript (length and sequence) were added to the sheep genome reference files used for bioinformatics analysis of sequence data alignment (Section 5.2.11) and the generation of expressed gene lists (Section 5.2.12). This approach allowed the ERCC transcripts to be subjected to the same experimental and normalisation procedures as the sample transcripts. Measured fragments per kilobase of exon, per million reads (fpkm) values for the ERCC controls could then be plotted against the known concentration of each ERCC transcript. Using an iterative approach, outliers with either low or high fpkm values were removed until an R^2 value of > 0.9 was achieved. This then determined the upper and lower limit of detection for gene expression in the fetal ovary dataset.

5.2.5 mRNA enrichment

RNA extracted from tissue includes only a small proportion of mRNA (<5%) (*299*). The majority of RNA is rRNA which is involved in translation but does not represent transcribed genes. mRNA indicates potential proteins synthesised by the fetal ovary. Additional RNA types present, but not of interest in this study include: transfer RNA, micro RNA, small interfering RNA, small nucleolar RNA, and Piwi-interacting RNA (*300*). rRNA must be removed from RNAseq samples so that informative mRNA transcripts can be measured at greater sequencing depths. rRNA can be removed either by rRNA subtraction, or enrichment of mRNA. Removal of rRNA is a more complex method and is generally used where the small RNA types are an important component of the study. In this study, mRNA enrichment was undertaken using the Dynabead mRNA Direct Micro kit (Thermo Fisher Scientific, Auckland, NZ), following the manufacturer's directions for samples containing 1 to 50 µg of RNA. The method utilises the polyA tail at the 3´ end of the mRNA. This tail binds to an Oligo dT sequence attached to a magnetic bead. A magnet is used to capture the Oligo dT/mRNA complexes from the total RNA solution. After a buffer wash to remove as much non-specifically bound rRNA as possible, mRNA is eluted from the Oligo dT/bead complexes by a 65°C incubation in 10mM Tris-HCl for 2 minutes.

5.2.6 RNA fragmentation

RNAseq platforms sequence relatively short lengths of mRNA, with the ideal length being platform dependent (*292*). In this instance, the platform used was the Ion Proton which can read fragments up to 200 bp in length. mRNA was fragmented with an RNaseIII digestion at 37°C (RNase III included in Ion Total RNA-Seq kit V2. Thermo Fisher Scientific, Auckland, NZ). A digestion time of 3 minutes was found to give mRNA fragments of the appropriate size. The distribution of fragment sizes was determined using the Bioanalyser (RNA 6000 pico kit, Agilent 2100, Agilent Technologies, Santa Clara, Ca, USA), as described in Section 5.2.2, at a dilution in nuclease free water of 1:20.

5.2.7 Library preparation

Current sequencing technologies are unable to directly sequence RNA fragments, therefore, mRNA is converted to cDNA followed by amplification prior to sequencing cDNA. Additionally, adaptors, barcodes, primer sites, and calibration sequences are all ligated to the cDNA fragments to make the sequencing library. Figure 5.4 illustrates the structure of an individual cDNA fragment following library construction with references to sections that explain the roles of each library adaptor component.

Libraries were constructed using the Ion Total RNA-Seq kit V2 (Thermo Fisher Scientific, Auckland, NZ) as per the manufacturer's instructions for fragmented poly (A) RNA up to 5 µg. A 3 µg aliquot of each mRNA enriched sample was used for library construction. The quality and concentration of each completed library was assessed with the Bioanalyser, using the Agilent DNA 1000 kit (Agilent Technologies, CA, USA), following the manufacturer's instructions.

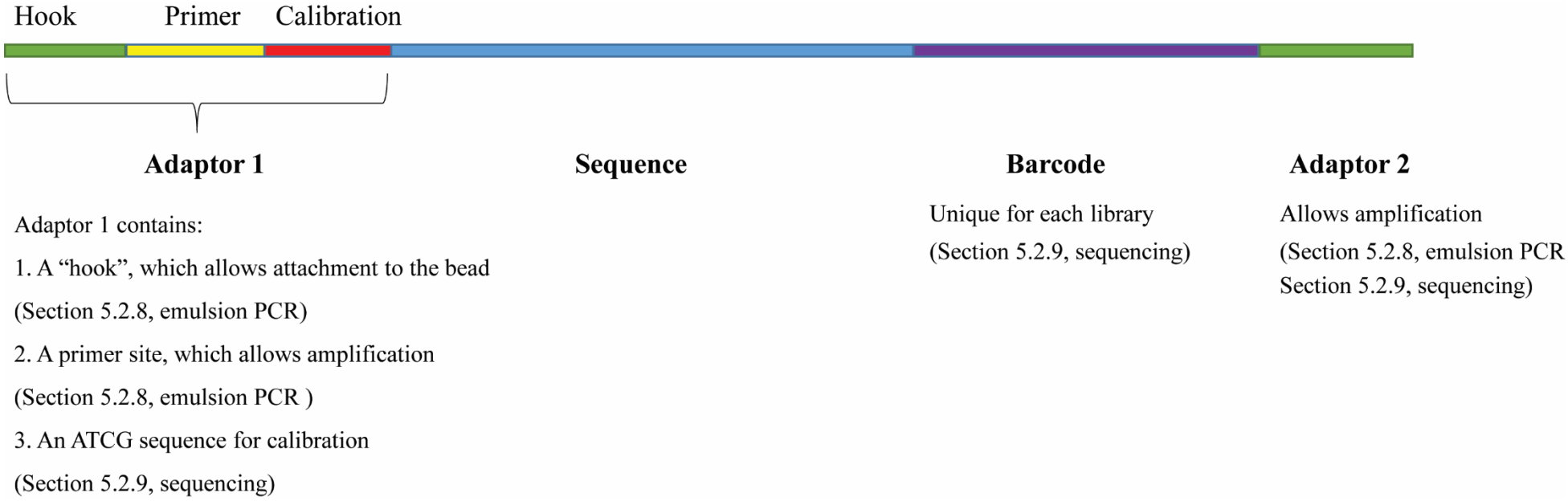

Figure 5.4 Components of each fragment of a completed cDNA sequencing library. References to thesis sections explains the functions of each added adaptor sequence.

5.2.8 Emulsion PCR and enrichment of Ion Sphere Particles

Emulsion PCR is the chemical cloning of individual library fragments and the attachment of numerous cloned copies of unique fragments to sequencing beads (Ion Sphere Particles or ISPs). This process is shown in Figure 5.5, and is reviewed by Kohn et al (*301*).

In Figure 5.5 A, an emulsion is created by the addition of oil, PCR reagents, Ion Sphere Particles (ISP´s), and a library sample. The purpose of the emulsion is to create aqueous droplets within which the chemical cloning reactions take place. The size of the aqueous droplets (termed micells or micro-reactors) in this emulsion are critical. This size distribution follows a Poisson distribution and is dependent on the ratio of the individual reagents, as well as the degree of agitation the emulsion is subjected to. The optimally sized micro-reactor will contain one ISP, one library fragment, and PCR reagents (Figure 5.5 B). The adapter ("hook") previously ligated to the 3′ end of the fragment, binds to a complimentary sequence with which the ISP is coated. Repeated PCR amplification cycles then detach the fragments, amplify them using the primer sites (also previously ligated to the library fragments) and reattach clones of the original fragment to the ISP (Figure 5.5 C). Following repeated cycles, the emulsion is broken using a detergent buffer, creating a suspension with millions of ISPs, each with up to 30,000 clones of a unique fragment (templates) attached (Figure 5.5 D).

In practice, most ISPs do not contain templates, so an enrichment step is required. A primer (complimentary to adapter 2, Figure 5.4) conjugated to biotin, binds to the end of the bead attached templates. Streptavidin coated magnetic beads are added (Figure 5.5 F), and following the streptavidin-biotin binding reaction, ISPs that are coated with template can be separated from the mixture magnetically. The template containing beads are centrifuged onto a multi-welled chip (Ion PI v2, Thermo Fisher Scientific) and subsequently sequenced. The goal is to achieve a chip that has a single ISP in each well. The sequence of the fragments (up to 30,000) in each well is identical, while the sequence of the fragments in individual wells differs from well to well. In reality, between 20 to 50% of wells contain more than one ISP, and these polyclonal wells are automatically removed from the analysis by the Ion Torrent software. The emulsion PCR and enrichment process were performed using the Ion One Touch 2 System and the Ion PI Template OT2 200 Kit v3, (Thermo Fisher Scientific, Auckland, NZ) following the manufacture's guidelines.

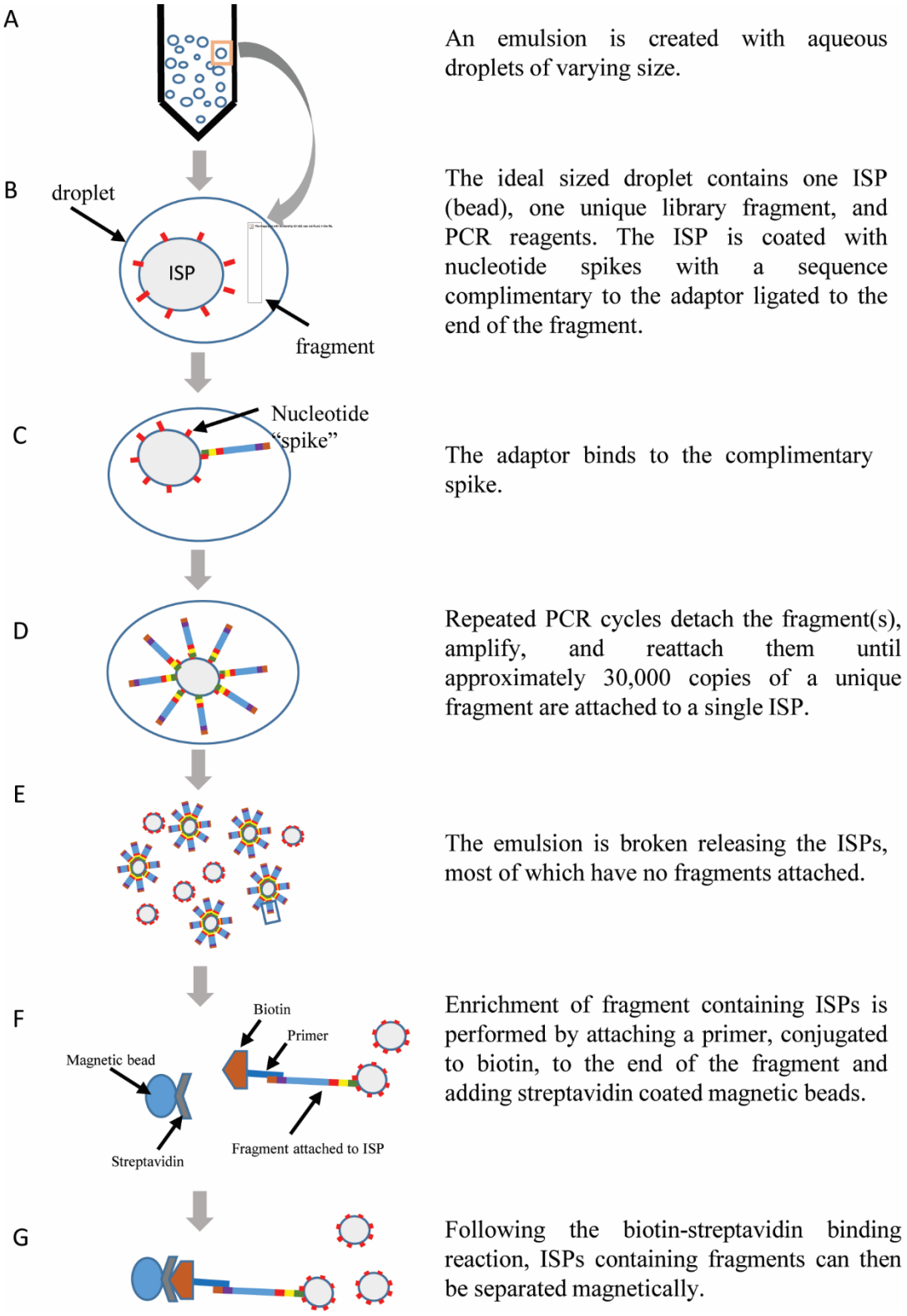

Figure 5.5 The concept of emulsion PCR.

5.2.9 Sequencing

Each RNAseq platform uses different technologies for sequencing. The Ion Proton technology used in this study is referred to as "sequencing by synthesis" and measures changes in pH when nucleotides are incorporated into a complimentary sequence. In each well of the Ion PI v2 chip, DNA strands are synthesised complimentary to the template fragments anchored to the ISP. The Ion Proton sequentially flows single nucleotides across the chip. If the next base in the template is complimentary to the base flowing over the chip, it is incorporated into the sequence. The incorporation releases an H^+ ion, resulting in a pH change, which is recorded by semiconductors at the base of each well. Each incorporation reaction occurs up to 30,000 times in each well, corresponding to the number of cloned fragments attached to the ISP.

For this study, the term Ion Proton run is defined as the sequencing from a single chip. The sequencing process is illustrated in Figure 5.6. The pH range for a single base incorporation for each well is determined using the calibration sequence (A-T-C-G) ligated at the beginning of each fragment template during the library construction process. As the number of fragments attached to the ISP may vary from well to well, calibration of each well is critical. The first bases sequentially added (T-A-G-C) are complimentary to the calibration sequence. This allows the instrument to calibrate the resultant pH changes for each well, for each of the four possible bases.

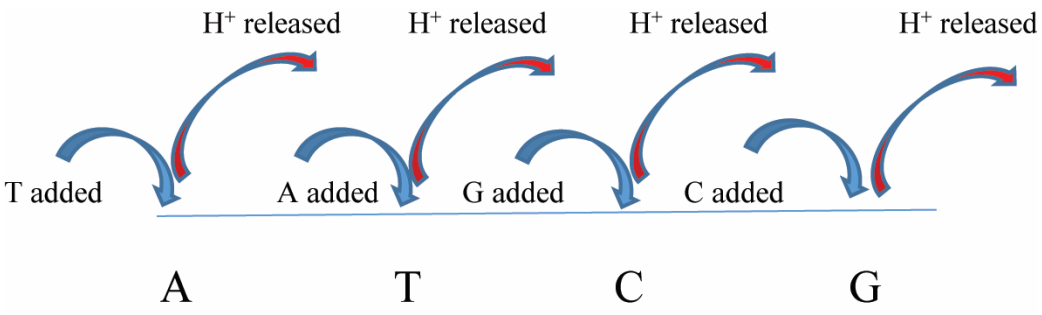

Figure 5.6 Principles of Ion Proton sequencing by synthesis. Individual bases are added sequentially and a complimentary DNA strand is synthesised. If the next available base in the fragment sequence is complimentary to the added base, incorporation occurs, which releases an H^+ ion resulting in a pH change which is detected by the instrument.

As sequencing progresses, subtle changes to the measured pH profile, reaction chemistry, and template integrity causing drift from the expected profile become apparent. These errors contribute to the generation of a Phred score for each base call.

Phred scores indicate the probability of each base call being correct and are presented on a log scale. A Phred score of 20 indicates a 99% probability of the base call being correct, while a score of 30 indicates a 99.9% probability of the base call being correct. It is usual for per base Phred scores to decline towards the end of each fragment read. In part, this is due to errors made during the sequencing process. For example, if a base call on an individual fragment is skipped, then that fragment becomes out of phase with the other fragments in that well. As these errors compound during the sequencing process and the number of fragments affected increases, then the probability of the base call being correct increasingly declines. Phred scores are based on platform dependent adaptations to the original methods described by Ewing et al (*302*).

The insertion of the library specific barcode during library construction allowed multiple libraries to be sequenced simultaneously (i.e. up to 12 libraries sequenced during each Ion Proton run) with data generated for each library being separated based on its barcode. Technical variation between each sequencing run could then be assessed by comparing data for each library from each of the runs in which that library was sequenced. Given the same starting quantity of cDNA library, each run should produce a similar number of reads. The same quantity of DNA from each library was added to each run i.e. a run of five libraries would contain 5 x 100 pmol/L. This means, all things being equal, each library in a run should produce a similar number of reads. In addition, read length and GC (guanine-cytosine) content are good indicators of sequencing quality (*303, 304*). Therefore, for each library, variation in read length and GC content between sequencing runs are appropriate measures of technical variation.

The outputs from a sequencing run are computer data files in FASTA format, where one file for each library sequenced in that particular run is generated. Beginning with these files, the remaining steps for analysing gene expression were computer based processing and analysis of the sequenced data output.

5.2.10 Pre-processing of reads following the Ion Proton sequencing

The main objective of pre-processing is to remove bases or complete reads with a low Phred score (< 20), particularly those bases toward the end of a sequence. Pre-processing is performed on each output file generated by the Ion Proton. If a library was sequenced in four independent runs, then the four output files were processed separately. For this study, the Fastx Tool Kit (v 0.0.14) was used, with two basic functions, Quality Filtering and Quality Trimming, being

utilised. Both functions utilise user defined variables p, q, and l (described below). To achieve optimum results, various combinations of values p, q and l were trialled with the optimum values (based on number of reads remaining, their length, and average Phred score) determined to be p = 40, q = 25, and l = 25. Filtering removed complete reads that have greater than p% of bases with a Phred score lower than q. Trimming trims sequenced reads on quality threshold. This tool trims the sequences from the end of a read if the quality of a base drops below the given threshold q. Trimming also removes sequences shorter than a pre-determined length l. The concept of pre-processing is a balancing act between retaining as many sequences (and sequence length) as practicable, while ensuring an adequate sequence quality.

It was thought that the order of application of these two functions (trimming and filtering) may affect the number and length of the reads remaining following pre-processing. By removing bases with a poor Phred score first (trimming), this would improve the % of bases (p value) remaining with the designated acceptable Phred score (q value). Therefore, fewer reads would be removed when subsequently filtering the data. To test this hypothesis, a comparison was performed where output files were trimmed then filtered, with these results compared to pre-processing by filtering then trimming. Results of this comparison are presented in Section 5.3.5.

5.2.11 Sequence alignment

Sequence alignment is a procedure where two or more sequences are compared to each other to determine if those sequences are homologous. In the context of this study, the comparison was between the sequences generated from the Ion Proton, and the sequences within the reference genome Ovis Aries 3.1 (OAR3.1, GCF_000298735.1) (*305*). Through this process, the data generated from the study becomes annotated, i.e. where reads align to a reference sequence known to be associated with a particular gene, the sequence is tagged with the associated gene identifier. Further, the number of reads which align to a given feature or gene gives an estimate of the abundance of that gene in the sample.

A number of software packages are available for alignment, and while each uses subtly different approaches, most rely on variations of the Smith-Waterman algorithm (*306*). In this study, a comparison of two software packages, Tophat (*307*) (v2.1.0) and STAR (*308*) (v2.3.0.1) demonstrated that STAR was the more efficient alignment package for this dataset (Section 5.3.5). Both Tophat and STAR also report the number of splice sites. These are defined as sequence elements in the mRNA that are recognised by splicing factors (*309*). The number of these sites gives an indication of the level of alternative splicing in the samples. Default settings for STAR were used with one exception, reads which mapped to more than 1 site in the reference genome were excluded from the output files.

5.2.12 Generation of gene lists using Cufflinks

To produce files of a manageable size, the Samtools (V 0.1.19-44428cd) software package was used to convert FASTA files from the Ion Proton output to SAM (sequence alignment map) files and subsequently compressed BAM (binary alignment map) files. Files corresponding to each individual library from different sequencing runs were then merged so that the data for each library (i.e. each fetal ovary) was contained in a single file. These files were then analysed using the Cufflinks software package (v2.2.1). Cufflinks takes the aligned reads and assembles them into individual transcripts, then matches these transcripts to specific genes within the reference genome. Where annotation data is available, it also identifies splice variants of the same gene. The expression level for each gene is calculated from the number of generated sequences that align to a gene.

The number of sequences that align to a gene is not solely dependent on the expression level of the gene but, based on probability, also upon the number of fragments in that library, and the length of the gene mRNA concerned. A library with a high number of sequences is more likely to have sequences align to a gene than a library with a low number of fragments. Additionally, longer sequences are more likely to align to a gene than shorter sequences. To allow for this, the raw number of aligned fragments is divided by the known length of the gene mRNA, then multiplied by the number of mapped reads from the library (*310*). The resulting value is fragments per kilobase of exon, per million reads (fpkm).

5.3 Results

5.3.1 Confirmation of fetal sex by PCR

While 24 samples were processed to the gene expression analysis stage, examination of the expressions profiles from each library revealed one outlier (day 75 restricted). For this sample, high expression of genes associated with testes development of *INSL3*, *INHA*, *INBA*, *STAR*, *HSD3B*, *CYP11A1*, *CYP17A1*, and *STAR* was noted. Similarly, low expression of oocyte genes such as *ZP2*, *ZP3*, *ZP4*, *GDF9*, and *FIGLA* were noted. PCR demonstrated the presence of a band at 527 bp in known male samples (fetal and adult), and also in the outlier sample. Sanger sequencing of these bands showed 100% homology with ovine *SRY*, thus confirming the outlier sample as a male (Figure 5.7). Results for this sample were removed from the remaining analysis for this thesis. Figure 5.7 shows an image from a DNA gel containing this sample (lane 3) illustrating the presence of SRY.

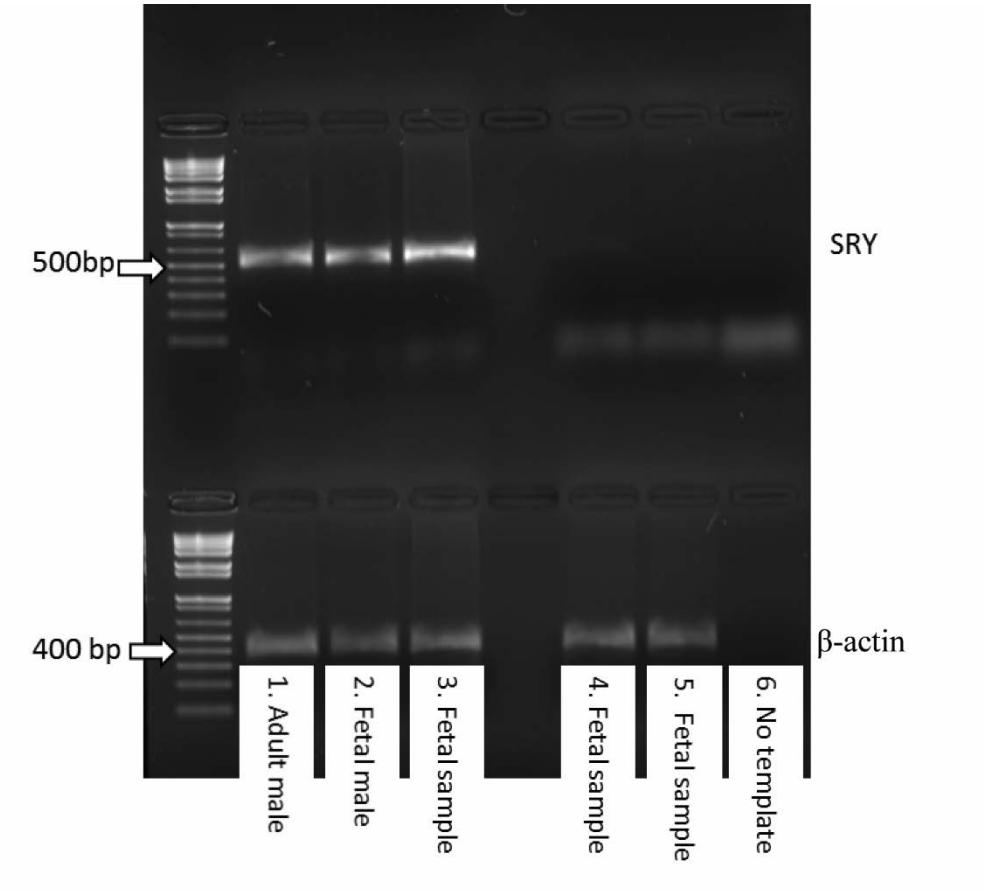

Figure 5.7 DNA electrophoresis gel showing sex determination using PCR. Upper gel shows SRY, lower gel shows β-actin. Lane 1 adult male control, lane 2 fetal male control. Lane 3 contains a sample thought to be female, but the presence of SRY confirms this sample as a male. Lanes 4 and 5 contain fetal samples confirmed as female. Lane 6 contains controls with no added DNA template.

5.3.2 RNA and DNA extraction

Nucleic acid extraction procedures were optimised using day 55 and 75 fetal testes (unless specified otherwise, Tables 5.1 and 5.2). Nucleic acid extraction with Trizol based protocols produced average RNA concentrations of 176 ng/µL with an average RIN value of 8.7 (Table 5.1). While RNA with these specifications is suitable for RNAseq work, the DNA contamination (as measured by Qubit assay) remained relatively high. Further, the techniques for extracting DNA from the samples did not prove effective, with no detectable DNA being present following extraction and purification.

Results obtained during optimisation of the Zymo Duet extraction protocol are shown in Table 5.2. Beginning with the manufacturer's recommended protocol (Table 5.2 Protocols A to C), the method was progressively refined until sufficient RNA and DNA of high quality was recovered. Protocols A to C show satisfactory RNA and DNA yields, however DNA contamination of the RNA samples prevented a RIN number being generated and indicated that these samples would not be suitable for RNAseq work. Serial elutions (Table 5.2 Protocols E and F, columns were eluted multiple times using fresh elution buffer for each elution) showed that a single elution was not sufficient to elute all the bound RNA and DNA off their respective columns. This result was reinforced by double elution results (Table 5.2 Protocol D, columns re-eluted with the initial flow through). The implication was that the columns were at their maximum binding capacity, allowing excess nucleic acid to flow through the columns. Increasing the extraction volume and processing multiple aliquots as individual samples (Table 5.2 Protocols H to K), not only increased the RNA and DNA yield (by providing multiple end samples), but also substantially reduced DNA contamination in the RNA sample.

Table 5.1 Results of Trizol based nucleic acid extraction procedures

Protocol	Age/Sex	RNA ng/µL Bioanalyser (total)	Final volume (µL)	RNA ng/µL Qubit (total)	RIN	RNA /mg	DNA in RNA ng/mL Qubit	DNA ng/µL Qubit
Trizol chloroform	55/Male	116.1	350	304	n/a	17.8	225	< 1
Trizol chloroform Qiagen RNA column	65/Female	421	50	200	9.0	9.0	4.83	< 1
Trizol chloroform Qiagen RNA column phase lock	55/Male	456	50	290	8.6	17.1	7.96	< 1
Trizol chloroform Qiagen column Manual homogenisation	55/Male	106	50	82	8.5	3.7	2.21	< 1
Trizol chloroform Zymo RNA column	55/Male	206	50	102	8.7	5.7	3.39	< 1

Table 5.2 Results of column based extraction procedures

Protocol	Method Synopsis	Age/sex	RNA ng/µL Bio analyser	RNA ng/µL Qubit	RIN	RNA /mg	DNA in RNA ng/µL	DNA
A	Manufacturer's protocol	55/M	576	282	n/c	22.8	823	187
B	Manual homogenisation	55/M	130	194	n/c	9.6	767	168
C	Ovary manufacturer's protocol	65/F	275	309	n/c	20.5	239	264
D	Double elution	55/M	862	434	8.4	28.9	386	81.6
E1	2 elutions, elution1	55/M	n/a	620	n/c	24.2	950	197
E2	2 elutions, elution2	55/M	n/a	295	n/c	11.5	437	62.4
F1	Serial elution1 (for both RNA and DNA)	55/M	840	738	n/c	32.1	472	802
F2	Serial elution2 (for both RNA and DNA)	55/M	760	574	n/c	25	580	282
F3	Serial elution3 (for both RNA and DNA)	55/M	38.6	44	8.5	1.9	19.7	55.6
F4	Serial elution4 (for both RNA and DNA)	55/M	27	<20	8.4	1.2	6.6	37.5
G	2x ex vol	55/M	428 (x2)	394 (x2)	n/c	19.7	218	63
H	4x ex vol (mean of 2 aliquots)	55/M	712	209	8.3	69.6	79.3	101
I	4x ex vol, DNase (mean of 2 aliquots)	55/M	404 (x4)	262 (x4)	8.8	21.8	53.3	91.4
J	2x ex vol (mean of 2 aliquots)	55/M	460 (x2)	184 (x2)	8.1	40.8	175	108 (x2)
K	2x ex vol DNase (mean of 2 aliquots)	55/M	538 (x2)	215 (x2)	8.3	47.6	107	81.6 (x2)
For the following trials, each sample/aliquot was centrifuged through 2 DNA columns, the first column retained for DNA extraction								
L1	4x ex vol, 2 elutions, elution1	55/M	287 (x4)	93 (x4)	9.7	37.2	4.3	94.4 (x4)
L2	4x ex vol, 2 elutions, elution2	55/M	nd	nd	nd	nd	nd	nd
M	4x ex vol, 2 elutions, DNase elution1	55/M	300	80	8.4	32.0	2.1	82.6
N	2x ex vol, DNase, double elution	55/M	435 (x2)	358	8.4	44	5.5	145 (x2)
O	2x ex vol, DNase x2, double elution	55/M	619 (x2)	233	9.95	52	4.4	145 (x2)

In the method synopsis column, double elution indicates the first eluent was re-centrifuged through the column yielding one sample. Two elutions indicates columns were eluted twice yielding two separate samples. Ex vol refers to the extraction volume, for example 2x ex vol indicates the sample was homogenised in 2x the recommended extraction volume with the sample subsequently split in two and each aliquot processed separately. DNase refers to the recommended in-column DNase digest, while DNase x2 refers to an in-column and a final DNase digest. nd indicates not detectable, n/a indicates not measured, and n/c indicates value not able to be calculated.

By centrifuging each aliquot from a single sample through two consecutive DNA columns (Table 5.2 Protocol L), DNA contamination was reduced further. Both an in-column DNase digestion (Table 5.2 Protocols N to O) and a DNase digestion of the final RNA sample (Table 5.2 Protocol O) ensured low DNA contamination. The results indicated that Protocol O produced the most efficient recoveries of both RNA and DNA. This protocol was then applied to the fetal ovary samples obtained at day 55 and 75 of gestation.

Using optimised Protocol O, a total of 30 fetal ovaries were used to extract nucleic acid for RNAseq library construction, with each ovary yielding 2x 50 µL RNA samples and 1x 50 µL DNA sample. The average concentrations of DNA and RNA recovered for each age and group are presented in Table 5.3. At day 75 of gestation, significantly more DNA was extracted from maintenance ovaries when compared to restricted ovaries of similar size ($p < 0.01$, ANOVA).

Table 5.3 Nucleic acid concentrations recovered from fetal ovaries using optimised protocol

Group	Ovary weight mg	RNA ng/µL	DNA contamination ng/µL	DNA ng/µL
Day 55 maintenance	10.3 ± 0.9	104.5 ± 13.5	< 12.8	103.6 ± 10.6
Day 55 restricted	10.1 ± 0.7	135.4 ± 18.7	< 11.8	109.0 ± 10.1
Day 75 maintenance	23.9 ± 1.3	332 ± 14.8	< 12.7	204.7 ± 15.0
Day 75 restricted	24.9 ± 1.3	302.1 ± 35.9	< 15.8	116.4 ± 10.8

Results are expressed as means and standard errors. For DNA contamination, most values were below the detection limit of the assay (10 ng/mL). For these samples, a value of 10 ng/µL was used to determine averages which were then expressed as < average value obtained.

5.3.3 mRNA enrichment, fragmentation and library preparation

Individual libraries were constructed from a total of 24 fetal ovaries (6 from each group at each age). The average RNA concentration and molarities for 23 of the completed libraries (as the male sample has been removed) are shown in Table 5.4 for each age and group.

Throughout the process, a Bioanalyser was used to control for the efficiency of RNA extraction by checking the concentrations and size range of total RNA, mRNA, fragmented RNA, and the completed cDNA library. Bioanalyser outputs are shown in Figure 5.8 illustrating the progression through these stages of a typical sample. For this example, 3 µg of total RNA produced 10 µL of mRNA at a concentration of 35.6 ng/µL, 7 µL of fragmented mRNA at a concentration of 13 ng/µL, and 15 µL of cDNA library at a concentration of 4 ng/µL. Figure 5.8 B shows that prior to fragmentation most mRNA has a length between 3000 to 4000 nt.

Following fragmentation, length was reduced to approximately 150 bp (Figure 5.8 C). This resulted in the cDNA library consisting of fragments < 200 bp in length (Figure 5.8 D), within the range for sequencing using the Ion Proton.

Table 5.4 Average library concentrations and molarities

Group	Library concentration ng/µL	Library molarity pmol/L
Day 55 maintenance	13.8 ± 1.9	130,023 ± 18,815
Day 55 restricted	13.5 ± 2.0	135,134 ± 17,086
Day 75 maintenance	14.5 ± 2.4	119,265 ± 17,932
Day 75 restricted	18.3 ± 1.9	165,325 ± 11,975

Values are means and standard errors.

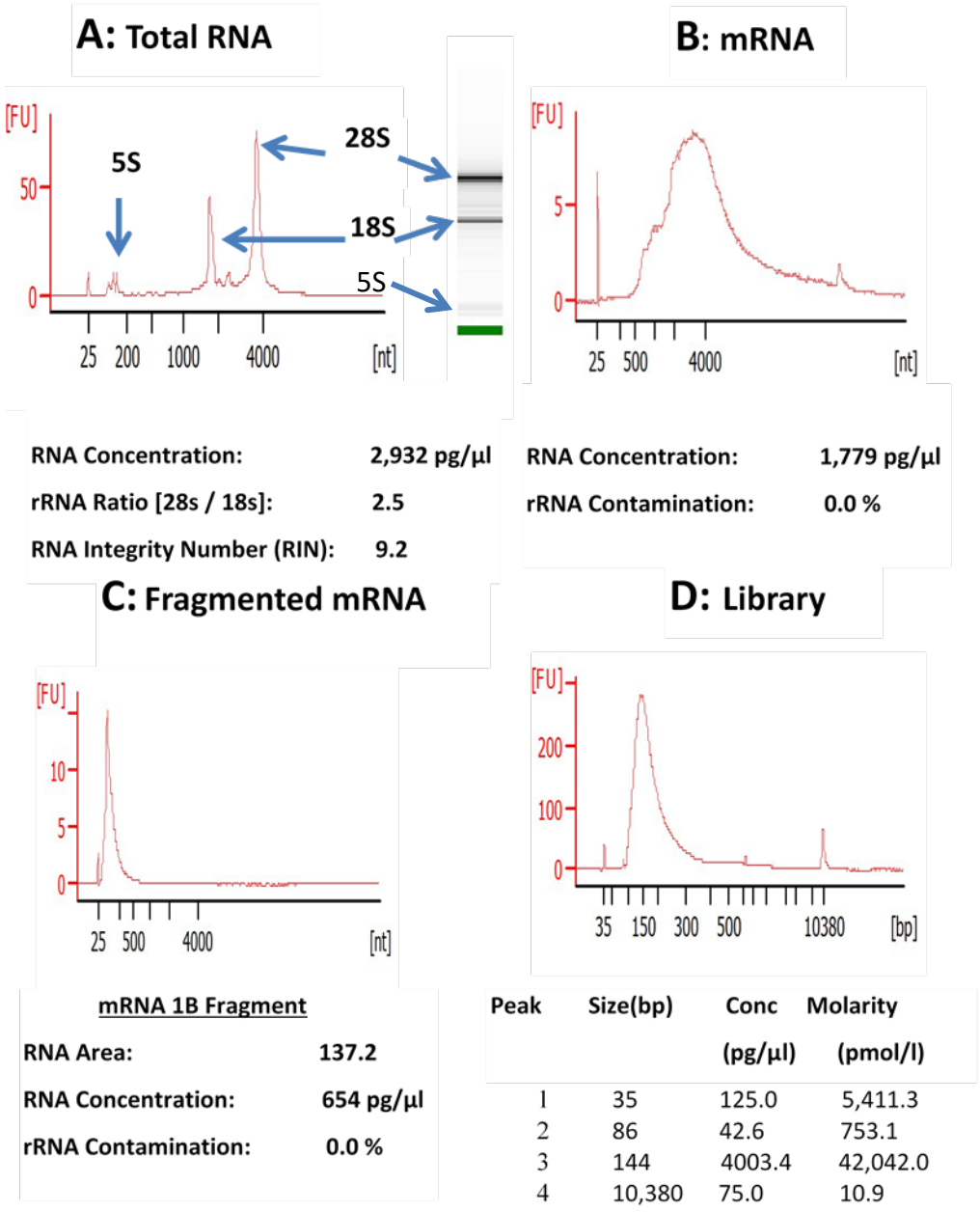

Figure 5.8 Total RNA to library Bioanalyser outputs for a typical sample. (A) Total RNA 1:200 dilution showing the predominance of 5S, 18S and 28S rRNA. The 28S:18S ratio gives an indication of the sample quality with a value of 2.4 being ideal. **(B)** mRNA 1:20 dilution following poly(A) enrichment. Most mRNA is greater than 2000 nucleotides in length. **(C)** Fragmented mRNA 1:20 dilution, most mRNA is now in lengths < 200 nucleotides following enzymatic fragmentation. **(D)** Completed library with fragment length averaging 150 bp. For this library, the concentration (the sum of the reported peaks, 2 and 3, lying between the marker peaks 1 and 4) is 4046 pg/µL or 42795 pmol/µL.

5.3.4 Sequencing of cDNA libraries

To reduce bias introduced by technical variation between individual Ion Proton runs (see Section 5.2.9), 15 separate sequencing runs were performed. Each run contained between four and 12 libraries, with the data generated for each library separated based on its unique barcode. Each library was sequenced in 3 to 4 separate Ion Proton sequencing runs and different combinations of libraries were run together. Total reads, average sequence length, and Phred score for each library is shown in Table 5.5.

For each library, the average between-run variation in read length was 5.0%, and the average variation in GC content was 4.0%. Each run generated an average of 59.3 million reads, with a between-run coefficient of variation of 4.0%. Within each run, the contribution of each library within that run varied by an average of 12%. Combined, this data illustrates minimal technical variation between sequencing runs.

Table 5.5 Sequencing data for each of 23 libraries

Library	Number of sequencing runs	Total reads	Average sequence length (bp)	GC content (%)	Average Phred score
1	4	32,841,230	72	51	24
2	4	30,659,552	90	50	24
3	4	41,021,163	102	51	24
4	4	39,843,036	85	50	25
5	4	38,562,731	95	50	24
6	3	36,100,278	84	48	24
7	3	37,453,728	69	51	24
8	3	43,055,898	109	51	25
9	3	48,833,617	70	48	24.5
10	3	37,396,828	110	48	24
11	4	36,417,753	64	49	24.5
12	3	37,526,440	81	50	24
13	3	34,683,455	112	50	24
14	4	33,572,057	72	48	24
15	4	33,916,331	74	46	24.5
16	4	41,548,809	70	49	25
17	4	35,057,583	67	49	25
18	4	43,452,171	95	48	25
19	4	31,816,559	69	48	24
20	3	35,793,410	83	49	24
21	3	37,175,362	79	48	24
22	4	36,794,741	85	49	25
23	4	35,408,631	89	49	25

5.3.5 Pre-processing of the sequencing data

Pre-processing involved the application of trimming and filtering algorithms to remove complete reads or individual bases with low Phred scores. Comparison of the order in which pre-processing was performed indicated that trimming followed by filtering had no effect on average read length or average Phred score for each library when compared to filtering followed by trimming. However, as hypothesised (Section 5.2.10), trimming followed by filtering yielded 2.8% more reads for subsequent alignment and analysis. This order of pre-processing was used for all data files, from all 23 libraries.

The effects of pre-processing on a typical read are presented in, Table 5.6 and Figure 5.9. Figure 5.9 illustrates pre-processing successfully resolved the issue of declining Phred scores towards the end of a read. In this example, from around 130 base pairs into the 270 bp read, Phred scores below 20 become increasingly apparent (Figure 5.9 A). Following pre-processing, the average read length was reduced to 230bp, but Phred scores remained above 20 throughout the length of the read (Figure 5.9 B). In Table 5.6 following pre-processing, the average number of reads for each library was reduced from 36.4 million to 24.5 million, the average read length dropped from 83 bp to 69 bp, and Phred score increased from 24 to 25.

Table 5.6 Effect of pre-processing on read characteristics for a single sequencing run

Protocol	Average number of reads	Average read length (bp)	Phred score
Before pre-processing	36,355,953	83	24
Post pre-processing	25,625,838	69	25

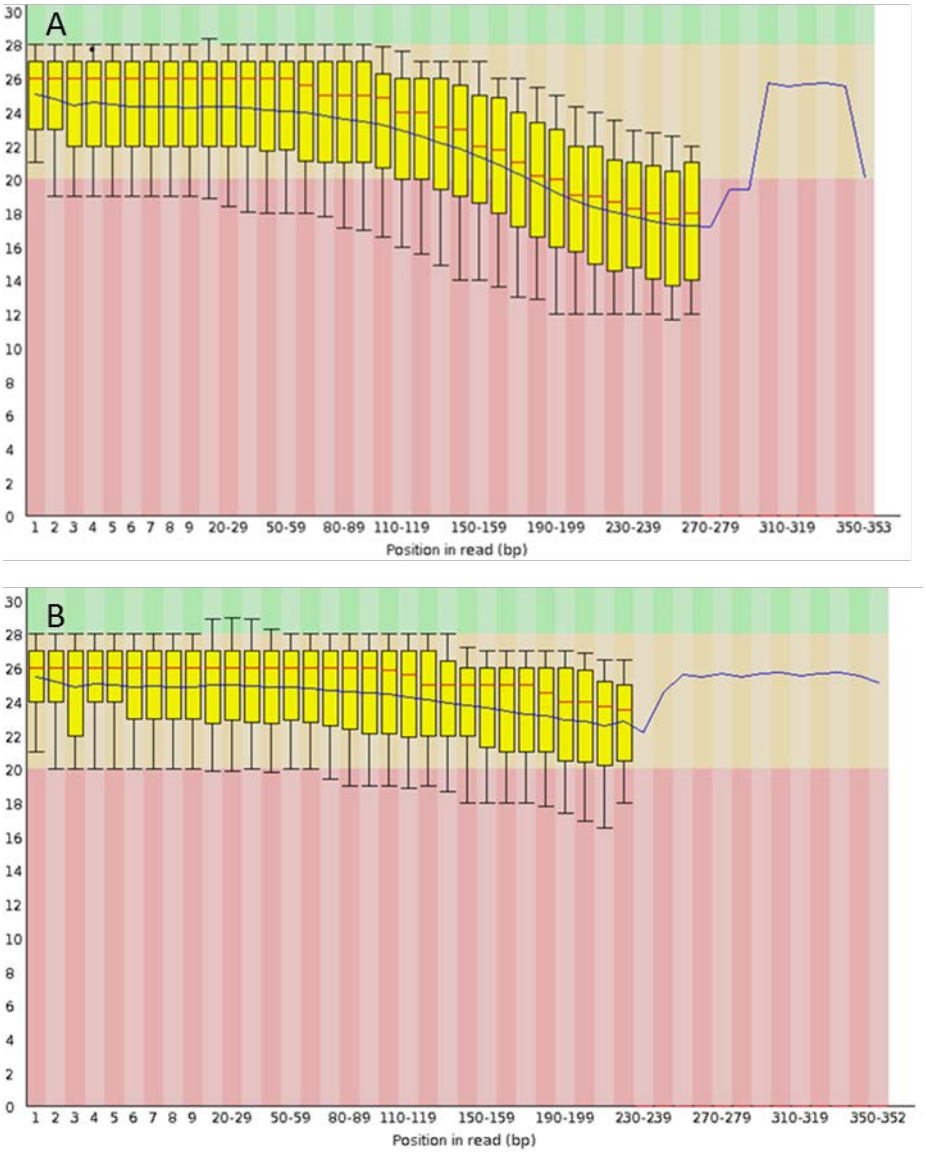

Figure 5.9 Effect of pre-processing on average Phred score. Graphs show Phred score on the vertical axis and position in the read (bp) on the horizontal axis. Central red line is the median value, the yellow box represents the inter-quartile range (25-75%). Upper and lower whiskers represent the 10% and 90% points. The blue line represents the mean quality. **(A)** Typical fall in Phred score towards the end of the read with a number of yellow blocks entering the red zone (Phred scores < 20). **(B)** Following pre-processing, while the read length is slightly shorter, those reads with a Phred score of < 20 have been removed with no yellow blocks entering the red zone.

5.3.6 Sequence alignment

As outlined in the introduction, sequence alignment involved aligning the reads from the dataset to the reference genome OAR3. Based on the literature, a target of at least 10 million aligned reads for each library was set to provide sufficient sequencing depth for differential gene expression analysis. Using a subset of the output files (one file from each library) from the Ion Proton, the efficiency of two alignment software packages (STAR and Tophat) were compared. For this comparison, default settings were used for both packages. STAR reported 147 million aligned reads to the reference database OAR3, while Tophat reported 120 million aligned reads. On this basis, STAR was used to align all files to the reference database.

Alignment data for each library is shown in Table 5.7. For each of the 23 libraries the alignment target of 10 million aligned reads per library was met, with an average of 18.4 million aligned reads per library being achieved. The average alignment was 71% of reads. While human (*311*) and rodent (*312*) datasets generally produce a higher percentage of aligned reads, the results obtained here are typical for ovine datasets (*313*) and merely reflect the level of annotation in the reference database. The number of annotated splice sequences was similar between all groups and ages, and this indicates there is no evidence supporting global differences in alternative splicing either between groups or between ages. The average per base mismatch rate over all libraries was 0.18%. This is an indication of the quality of the libraries as the STAR user's manual states a value < 0.8% indicates a library of good quality.

Table 5.7 Alignment data for individual libraries

Age	Group	Mapped reads (unique)	Splices (annotated)	% splices per mapped reads	% mapped
55	M	20,490,076	5,267,172	26	78.66
55	M	17,017,427	4,744,158	28	72.04
55	M	21,622,908	5,230,303	24	77
55	M	19,010,334	4,323,653	23	76.79
55	M	17,716,890	3,224,167	18	69.1
55	M	17,567,118	3,418,153	19	74.03
55	R	16,625,245	4,192,715	25	55.57
55	R	20,740,905	5,901,219	28	74.62
55	R	16,742,319	3,343,464	20	79.79
55	R	19,595,662	4,379,325	22	75.32
55	R	26,701,979	8,736,796	33	71.5
55	R	16,452,178	3,585,972	22	76.4
75	M	15,842,191	5,229,425	33	76.28
75	M	24,210,139	4,745,838	20	68.42
75	M	17,186,999	3,622,344	21	63.06
75	M	14,083,531	4,297,927	31	62.29
75	M	18,115,690	3,554,793	20	71.93
75	M	12,244,221	3,899,005	32	67.2
75	R	18,099,890	4,679,378	26	70.33
75	R	20,826,235	4,777,067	23	75.2
75	R	17,583,577	4,588,105	26	67.64
75	R	23,058,823	4,190,384	18	73.48
75	R	16,775,211	3,428,710	20	66.59

5.3.7 Determination of the lower limit of detection (LLD) from the ERCC spike-in controls

ERCC controls spiked into each total RNA sample during library construction were processed so limits of detection of the experimental data could be interpreted with confidence. The average measured fpkm value for each detectable ERCC transcript was plotted against the known concentrations of that transcript. Outliers were removed from the analysis until an R^2 value of > 0.9 was achieved. Transcript ERCC0074 consistently returned the highest fpkm reading, being at least 2 fold higher than the next highest value. For fpkm plots to achieve an R^2 value > 0.9, it was necessary to remove this value from all libraries. When raw counts were plotted against the known transcript concentration, removal of the value for transcript ERCC0074 did not affect the R^2 value (Figure 5.1 A). This implies that the normalisation procedures have a major impact on the fpkm values calculated for ERCC0074 and this impact will be explored in the discussion.

Figure 5.10 shows the edited average fpkm values for each transcript plotted against the known concentrations of the same transcript. In this plot, all measured fpkm values < 0.1 were removed to achieve an R^2 value > 0.9. The only value > 0.1 which was removed was the value for ERCC0074. The linear relationship achieved between measured and known concentrations indicates that for assessing gene expression in this dataset, fpkm values > 0.1 are a reflection of transcript concentration and therefore represent real expression.

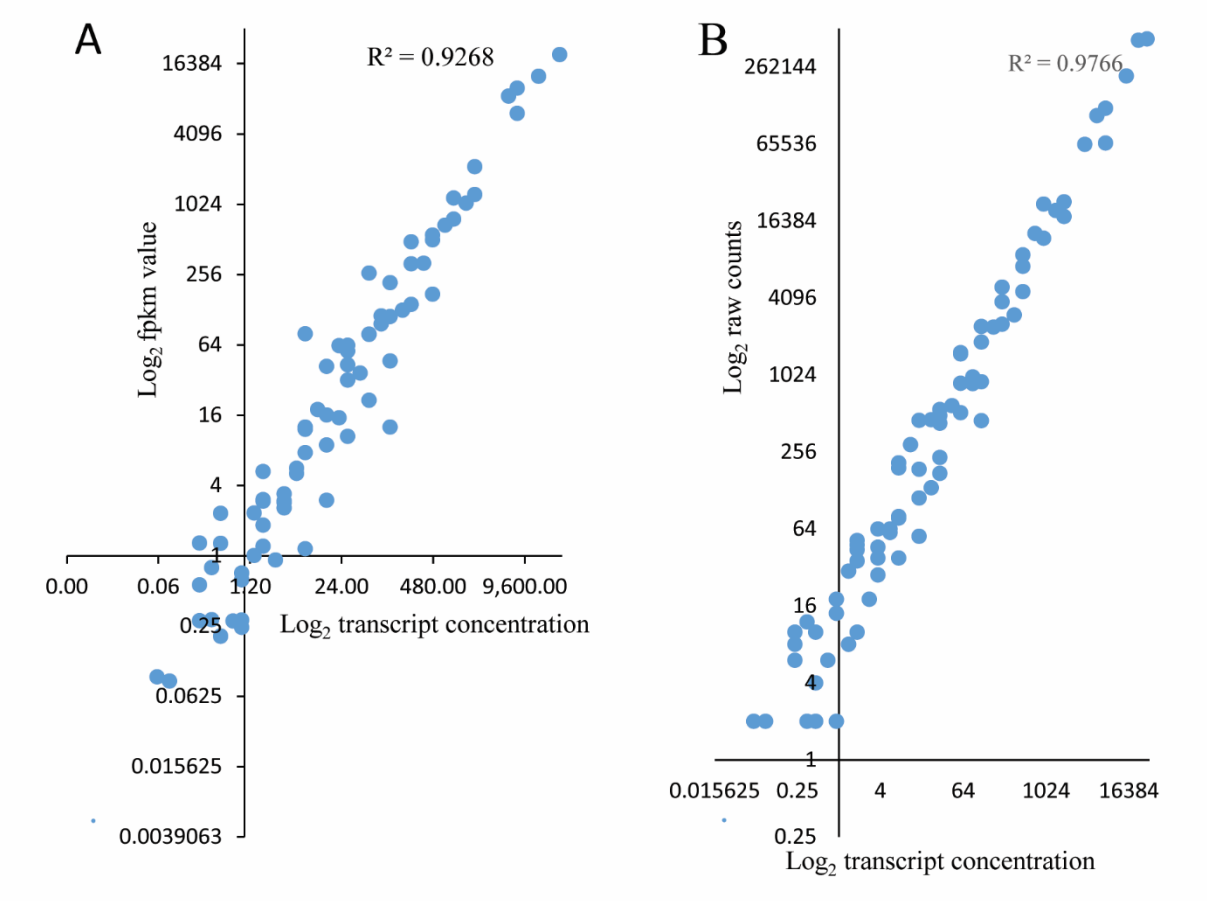

Figure 5.10 Concentrations of ERCC transcripts for a typical library. (A) Plot of unedited raw counts plotted against known concentrations. An R^2 value > 0.9 was achieved without removal of the data for the highest measurable ERCC transcript. **(B)** Plot of edited fpkm values plotted against known concentrations. Outlier values have been removed to achieve an R^2 value > 0.9.

5.3.8 Generation of gene lists using Cufflinks

The number of transcripts identified by Cufflinks with an fpkm value > 0.1 are presented in Table 5.8. The aligned transcripts are split into those that align to known genes and those that align to unannotated sequences within the reference database.

Irrespective of nutritional group, for fetal ovaries at day 55 of gestation, 61% of aligned transcripts had an expression level > 1 fpkm, a cut off value commonly used in RNAseq studies. Using the LLD indicated from analysis of the ERCC transcripts, 99% of aligned transcripts showed an expression level > 0.1 fpkm. At day 75, 66% genes had an expression level > 1 fpkm, and 98% showed an expression level > 0.1 fpkm. The distribution of fpkm values at both day 55 and 75 is presented in Figure 5.11.

Table 5.8 Number of aligned transcripts with expression > 0.1 fpkm

Age	Genes with fpkm > 0.1		Unannotated genes with fpkm > 0.1	
	Maintenance	Restricted	Maintenance	Restricted
Day 55	14,834	14,530	3,406	3,788
Day 75	15,038	15,009	3,567	3,562

Applying the criteria that fpkm values > 0.1 represent true expression and 0 fpkm represent no expression (values in between 0 and 0.1 could potentially be either no expression or low expression), then the number of transcripts that are either switched on or off between day 55 and day 75 of gestation can be estimated. These transcripts are presented in Table 5.9.

Table 5.9 Number of transcripts switched on or off between day 55 and day 75 of gestation

Change in expression from day 55 to day 75	Maintenance	Restricted
Switched on from fpkm 0 to fpkm > 0.1	476	401
Switched off from fpkm > 0.1 to fpkm 0	387	390

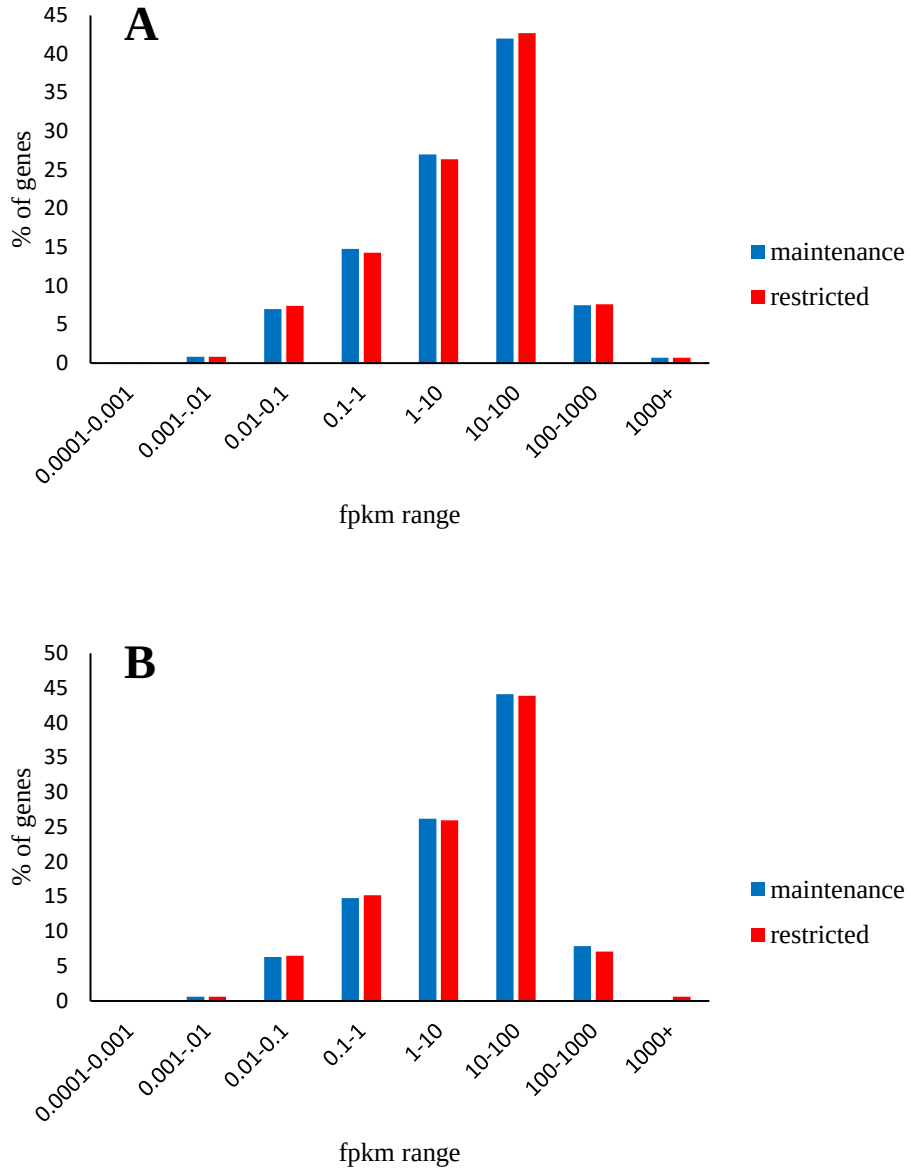

Figure 5.11 Distribution of expression values. Expression values (fpkm) are for transcripts with expression values > 0 fpkm. **(A)** Day 55, **(B)** Day 75.

5.3.9 Expression levels of autophagy related genes

Prior to differential gene expression analysis, the data generated in this chapter has the potential to provide insights into the metabolic and functional activities with the fetal ovaries. While in general these are not directly related to goals of this study, one relevant example is the expression of autophagy related genes. Tsujimoto and Shimizu proposed the relative expression of the autophagy associated genes *ATG5* and *ATG6*, when compared to other autophagy related genes, are an indicator of the functional role of autophagy in a given tissue (*293*). Where autophagy is involved in cell death, the expression of *ATG5* and *ATG6* are highly up-regulated compared to other autophagy related genes. To clarify the MAPLC3 IHC results (Section 4.3.4), the RNAseq data related to autophagy associated genes are presented in Table 5.10. Relative to other autophagy related genes, *ATG5* and *ATG6* are not highly up-regulated. Based on this result, and the work of Tsujimoto and Shimizu (*293*), it is likely that autophagy in the day 55 and day 75 fetal ovaries is playing a role in cell survival rather than cell death.

Table 5.10 Expression levels of autophagy associated genes in fetal ovaries

	Day 55		Day 75	
Gene	**Maintenance fpkm**	**Restricted fpkm**	**Maintenance fpkm**	**Restricted fpkm**
MAPLC3	392	434	483	545
ATG5	9	9	10	9
ATG6	61	63	60	57
ATG10	15	13	14	12
ATG12	34	32	38	32
ATG3	84	88	74	84

Values in red relate to genes reported as highly up-regulated during autophagic cell death. Expression levels of *ATG5* and *ATG6* are similar to the other autophagy related genes suggesting that autophagy is playing a role in cell survival.

5.4 Discussion

Based on the assessment of RNA quality, library quality, sequencing data, alignment data, and the LLD as determined by analysis of ERCC transcript expression levels, the data obtained is of high quality and ideally suited for analysis of gene expression patterns. While this chapter is predominantly focused on technical aspects of RNAseq, the data generated gives insights into the biological processes within the fetal ovary.

The finding of one male sample as indicated by PCR was unexpected. This sample was derived from a set of twins, one female and one male. The weight data for this gonad clearly indicated the sample as female, histological sections of the contralateral gonad also confirmed the presence of an ovary. Given the PCR result and the pattern of gene expression, the conclusion is that this resulted from an error in sample labelling of frozen gonads at the time of collection. PCR confirmed the sex of the remaining samples as female. It is important to note that this error had no impact on the results presented throughout this thesis as data from this sample was removed from all analysis.

The control of variation, both technical and biological, in RNAseq studies is an important consideration. Analysis of the number of reads generated for each sequencing run, read length, and GC content following sequencing, indicates a low level of technical variation in this dataset.

Biological variation (variation between individual animals) is controlled for by arguably, the most important considerations in designing an RNAseq experiment: the number of biological replicates to use, and the number of aligned reads achieved per sample (sequencing depth). Recommendations vary between studies and depend on the software being used for analysis, the goal of the study, and the fold changes in gene expression being targeted. In experiments where fold changes of two or more are the goal, Schurch and colleagues recommend a minimum of three biological replicates (*314*), with this number increasing for more sensitive requirements. Overall, Schurch and colleagues recommend six biological replicates as suitable for most studies. Using a human cell line, Liu and colleagues show a trade-off between biological replicates and sequencing depth (defined as the number of aligned reads). Lui and colleagues showed that increasing sequencing depth beyond 10 million aligned reads per sample gives diminishing returns on the ability to detect differentially expressed genes, whereas addition of biological replicates improves this return (*315*). Further, they contend that a sequencing depth of 10 million reads for between two and six biological replicates is sufficient in the majority of cases. Diminishing returns beyond 10 million reads was also reported by

Wang and colleagues, although increasing to 30 million reads allowed detection of all annotated genes compared to 80% of genes at 10 million reads (*316*). A similar broad conclusion was also reached by Hart and colleagues (*317*). The current study is based on five or six biological replicates per group with an average of 18.4 million aligned reads per library, which clearly meets the recommendations from the literature.

The range of fpkm values that represent real expression varies between studies. As a general guide, provided that sequencing depth is adequate (minimum of 10 million aligned reads per sample), a conservative cut off value of 1 fpkm is often used, although values as low as 0.2 fpkm have been shown to represent true gene expression (*318*). In the current study, analysis of the ERCC control mixes indicates that values of 0.1 fpkm show a linear relationship with transcript concentration and therefore are likely to represent real gene expression. In this dataset < 1% of fpkm values fall below 0.1 fpkm.

The analysis of the ERCC transcript levels also highlighted an issue with ERCC0074, the ERCC transcript which consistently returned the highest fpkm values. The fpkm values generated for this transcript did not fit the linear relationship between measured fpkm values and transcript concentration. However, the raw counts (non-normalised) did appear to fit the relationship suggesting that the normalisation procedures used by the software had a major impact on the fpkm values for this transcript. The Cufflinks software (and CuffDiff used in Chapter 6) apply a number of normalisation procedures to the raw data to generate fpkm values. One of these normalisation procedures is the application of a scaling factor to highly expressed genes, as results for these highly expressed genes can skew the data for those genes with low expression (*319*). Given that fpkm values for ERCC0074 were two fold higher than any other transcript, it is thought that this normalisation procedure may be contributing to the non-linearity of this transcript.

The number of transcripts that are switched on or off between day 55 and day 75 likely indicates the changing developmental status of the fetal ovaries as outlined in Section 1.1. For example at day 75, dissolution of ovigerous cords and formation or follicles is observed, processes not apparent at day 55.

A relevant example of how the data generated in this chapter (without progressing to differential gene expression analysis) can add to a study is the expression levels of the autophagy related genes. Analysis of the autophagy associated genes *ATG5* and *ATG6* provides evidence that at the gestational ages studied, autophagy is playing a role in germ cell survival and not cell death,

based on the work of Tsujimoto and Shimizu (*293*). This result is consistent with the IHC data reported in Section 4.3.4 and supports the findings of Gawriluk and colleagues (*289*). While autophagy appears not to be affected by restricted gestational nutrition, the data adds another dimension to the debate surrounding the role of autophagy in the developing ovary.

In summary, the RNAseq techniques applied in this study have generated quality data with high sensitivity ideally suited for the analysis of differential gene expression which will be presented in Chapter 6.

Chapter 6 . Differential Gene Expression in Fetal Ovaries

6.1 Introduction

To understand the potential mechanisms underlying the effects observed in this study to date, the data generated in the Chapter 5 has been used to analyse differential gene expression in fetal ovaries exposed to restricted gestational nutrition. Nutrition is known to regulate gene expression through different mechanisms including transcription initiation, RNA splicing, and mRNA stabilisation (*291*), all of which may be reflected in RNAseq results.

The mechanisms underlying the effects observed in the current study, and also in the literature, remain elusive. The evidence implicates germ cell development, a complex process involving a multitude of genes and pathways. Additionally, it remains unclear whether the nutritionally induced effects act directly on the germ cells or are mediated via pre-granulosa cells or other ovarian cell types.

While the data generated in the current study is from whole fetal ovaries, a wealth of literature is now available describing the cell specific expression pattern of genes in the fetal ovary. The most comprehensive of these studies describes the expression pattern of over 12,000 genes in the developing mouse ovary (*320*). This study used transgenic mouse lines with fluorescent reporters to enable fluorescent cell sorting of digested ovaries to separate germ cells, supporting cells, stromal cells, and endothelial cells. Microarray analysis was performed to examine gene expression in each cell population. Extensive use of data from the developing mouse ovary (*320*) is made throughout this chapter.

The use of RNAseq to study the effects of nutrition on ovarian development has been reported in one other study to date. In cattle, Costa and colleagues reported nutrition induced changes to the transcriptome of the fetal ovary (*174*). While these changes to the transcriptome supported developmental changes to the ovary, the functional implications of the changes were not discussed. The possibility of relating changes in gene expression to morphological, endocrine, and physiological changes already observed in maternal animals, fetuses, and offspring from the same cohort of animals, highlights the advantage of the multi-disciplined approach adopted in this thesis. The prospect of relating nutritionally induced changes in gene expression to functional changes is enhanced by the use of pathway analysis (IPA, QIAGEN Redwood City, USA) and Gene Ontology (GO) terms (*321*). Identification of affected pathways may lead to

strategies targeting these pathways to either overcome the negative effects of under-nutrition, or to induce the positive effects observed in this study.

Therefore, the aim of the work described in this chapter was to use RNAseq to identify changes in gene expression and functional pathways in fetal ovaries exposed to restricted gestational nutrition. New hypotheses to explain the observed relationship between gestational nutrition, ovarian development, and postnatal fertility will then be developed from this data.

6.2 Materials and methods

6.2.1 Detection of differential gene expression between groups

The Cuffdiff (*307*) (v 2.2) software package was used to determine those genes differentially expressed between groups at each gestational age studied. Before calculating fold changes between groups and assigning significance to these changes, Cuffdiff first applies a number of normalisation procedures to the data (*307*). Firstly, as described in Section 5.2.9, to allow for variations in the number of fragments within a library, and variations in transcript size, raw data is converted to fpkm values. Secondly, a common feature in RNAseq data is that a small fraction of highly expressed genes usually accounts for large proportions of the sequenced reads. Further, small expression changes in these highly expressed genes can skew the results for genes with low expression. To address this, a scaling factor is applied based on number of aligned reads. For each library the median read count is divided by the geometric mean for all genes (*319*). To address an issue termed over dispersion (where the variance in gene expression between biological replicates is often greater than the mean expression level for that gene), Cuffdiff models the data to a negative binomial distribution (*319*). Finally, tests for significant fold changes between groups are based on t tests. As thousands of between-group tests are performed (in this case over 16,000), using a p value to indicate significance would result in a high false discovery rate (FDR). Cuffdiff utilises the Benjamini-Hochberg procedure (*322*) to manage the FDR and control the expected proportion of tests which reject the null hypothesis (i.e. that there is no difference between treatment groups). Ultimately, significance is assigned based on a q value rather than the p value generated from the t test.

Figure 6.1 illustrates the effect of applying the Benjamini-Hochberg procedure to the day 55 gene expression data. The number of differentially expressed genes (red dots, $p < 0.05$ for A and $q < 0.05$ for B) is substantially reduced following removal of what are deemed to be false positive results using the Benjamini-Hochberg procedure.

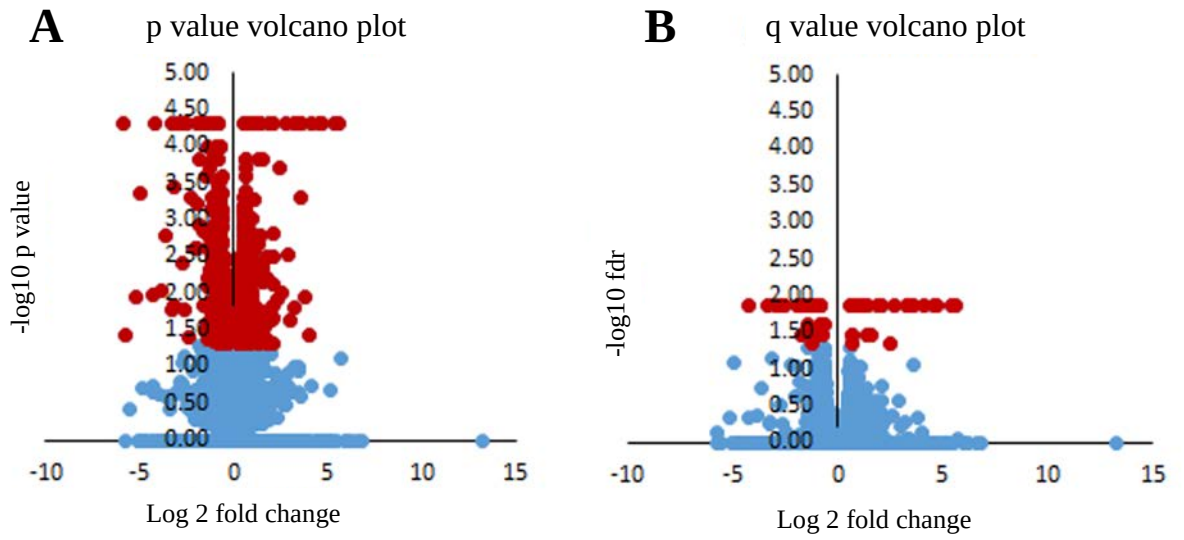

Figure 6.1 Day 55 volcano plots illustrating effect of FDR procedures. (A) Differentially expressed genes (red dots) are reported using an unadjusted p value ($p < 0.05$). **(B)** Differentially expressed genes are reported following application of a FDR procedure and using an adjusted p value (q value) of $q < 0.05$ to indicate significance.

6.2.2 Data analysis

To determine potential biological pathways, and the functional significance of differential gene expression reported by Cuffdiff, three approaches were used for analysis: Ingenuity Pathway Analysis software (IPA, QIAGEN Redwood City, USA), GO terms (*321*), and literature searches.

IPA was employed to examine pathways affected by differential gene expression. In combination with the q value, IPA also utilises the magnitude of fold changes between groups, the number of genes associated with the pathway being analysed, and current knowledge relating to the pathway concerned (e.g. rate limiting steps) to give strength to its pathway predictions. Results are expressed as p values and activation (z) scores. The p values are based on the number of focus genes (genes with a significant fold change between groups based on the q value) in the dataset, and the total number of genes known to be associated with that function or pathway. A significant p value for a given pathway indicates that the pathway has more focus genes associated with it than would be expected by chance. The z score is similar, but takes into account the direction of fold changes in the data, and compares this to information from the literature regarding the dynamics of the pathway concerned. A z score of < 2 indicates

that the pathway is likely to be down-regulated, while a z score of > 2 indicates the pathway is likely to be up-regulated. IPA classifies pathways as either canonical or functional. In general, a canonical pathway encompasses a broad category e.g. acute phase response signalling, whereas, functional pathways are more defined e.g quantity of oocytes. IPA also includes an extensive reference database describing the roles of each pathway, and unless specifically referenced, functional descriptions of pathways have been sourced from this database.

Some aspects of the data required adjusting before IPA analysis. Where the expression value reported by Cuffdiff for one group was 0, and the second group being compared was reported as > 1, Cuffdiff reports the fold change as infinite or –infinite (as the denominator in the calculation is 0). Based on bioinformatics advice (Mr Paul Maclean, AgResearch, Lincoln), where instances such as this occurred, an arbitrary value of 100 was assigned to the fold change so that the data was compatible with the software packages used.

Where available, GO terms have been assigned to genes of interest. GO terms describe what is known about a gene or a gene product. GO terms fall into three major categories: molecular function, biological processes, and cellular components. Terms are based on published literature and computer projections based on the known structure of the molecule. For this study, GO terms were largely sourced using AMIGO2 (*321*). An extensive literature search was used to provide further information regarding roles of genes and pathways of interest in fetal ovarian development.

6.2.3 Validation of RNAseq data

Nanostring technology was employed to validate the differential gene expression data obtained from the RNAseq procedures. In contrast to RNAseq which uses mRNA and several normalisation steps for raw counts (i.e. number of transcripts aligned to a gene), Nanostring uses total RNA and results are direct counts of identified gene sequences normalised to housekeeping genes.

The concept of Nanostring is outlined in Figure 6.2. A code set is produced comprising of a reporter probe and a capture probe. Each probe recognises a complimentary, adjacent, 50 bp sequence of the RNA transcript of interest. Both probes are hybridised to the sample RNA forming a complex. The reporter probe has a sequence of coloured fluorescent tags attached, with the order of these coloured tags uniquely identifying the transcript (gene) of interest. The purification sequences (one for all reporter probes, and one for all capture probes) are used to bind the probes and complexes to magnetic beads with complimentary sequences. Two

purification steps are then performed. Firstly, beads with nucleotides complimentary to the capture probe purification sequence are added. This binds both the probe/transcript complexes and the excess capture probes. The excess reporter probes are removed. Secondly, magnetic beads with nucleotides complimentary to the reporter probe purification sequence are added. These bind only the probe/transcript complexes, and excess capture probes are removed. The purified probe/transcript complexes are then eluted off the beads. The capture probes have a biotin moiety attached which is used to anchor the complexes to a streptavidin coated glass plate during an electrophoresis step. This process straightens the probe/transcript complexes so that the instrument (nCounter *MAX*, Nanostring Technologies, Seattle, WA, USA) can recognise and count the number of complexes based on the number of specific fluorescent tag sequences retained on the capture plate.

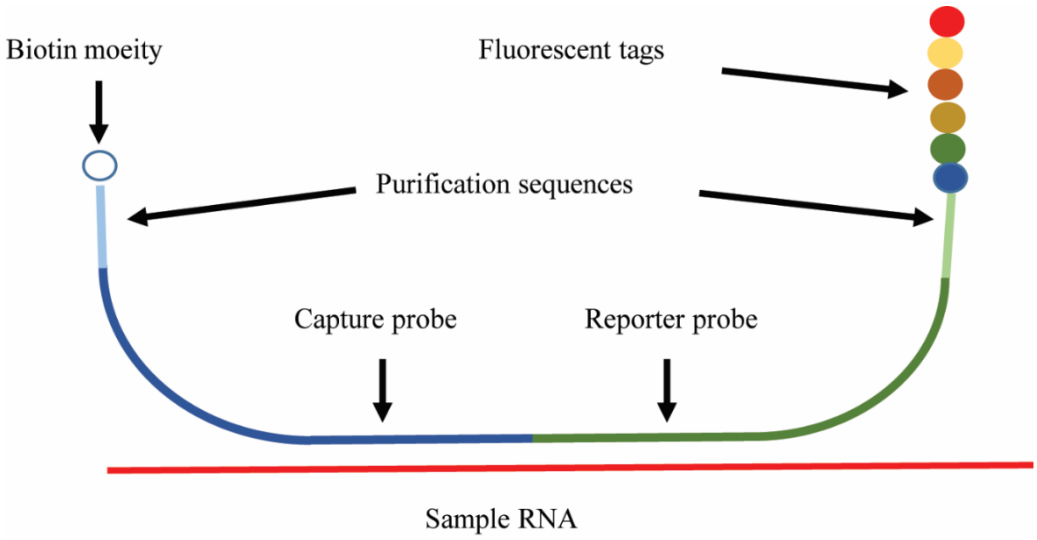

Figure 6.2 Concept of Nanostring technology. Reporter and capture probes bind to complimentary sequences of the gene of interest. Following purification and electrophoresis, the probe/gene specific sequence of fluorescent tags are counted by the instrument using a microscope and high definition camera.

For this study, the Nanostring nCounter XT gene expression assay was used (Nanostring Technologies, Seattle, WA, USA). The manufacturer's protocol was followed using 2 µL of total RNA. This protocol was then repeated using 4 µL of total RNA. Genes considered below the detection limit of the assay failed to show an increase in counts when the higher quantities of RNA were used in the assay. Counts were normalised to housekeeping genes and analysis performed using the Nanostring N solver software (v2.6). Based on RNAseq gene expression data, genes with a range of fold changes, a range of significance levels associated with fold changes, a range of expression levels, and genes of particular importance in ovarian development were selected for Nanostring analysis. A total of 48 genes were selected which included the housekeeping genes *YWHAZ, SDHA, GAPDH*, and *G6PD*. Code sets (reporter and capture probes) were synthesised by Nanostring Technologies. A full list of genes and target sequences can be found in Appendix G. The target sequences are 100 bp in length, 50 bp being complimentary to the reporter probe and the remaining 50 bp complimentary to the capture probe.

6.3 Results

Throughout this section, numerous references are made to fold changes in gene expression. Fold changes refer to expression levels in restricted fetal ovaries when compared to maintenance fetal ovaries. Therefore, up-regulation, or a positive fold change, indicates higher expression levels in restricted ovaries compared to maintenance ovaries. Conversely, down-regulation, or a negative fold change, refers to lower expression in restricted ovaries compared to maintenance ovaries.

6.3.1 Analysis of ERCC control mixes for fold change validation

As described in Section 5.2.4, two separate ERCC control mixes were added to the total RNA. The two mixes contain identical transcripts, but at different concentrations. Comparison of the known fold change in concentration between the two mixes compared to the measured fold change indicates the accuracy of the measured fold change. Plotting known fold changes against measured fold changes (Figure 6.3) should result in a linear relationship with an R^2 value > 0.9. Repeating the procedure described in Section 5.2.4, beginning with values < 0.1, values were removed until an R^2 value > 0.9 was achieved. To achieve the result illustrated in Figure 6.3, all values < 0.3 fpkm were removed. This indicates that for reliable fold change comparisons as low as 1.5 fold in this dataset, fpkm values > 0.3 are required.

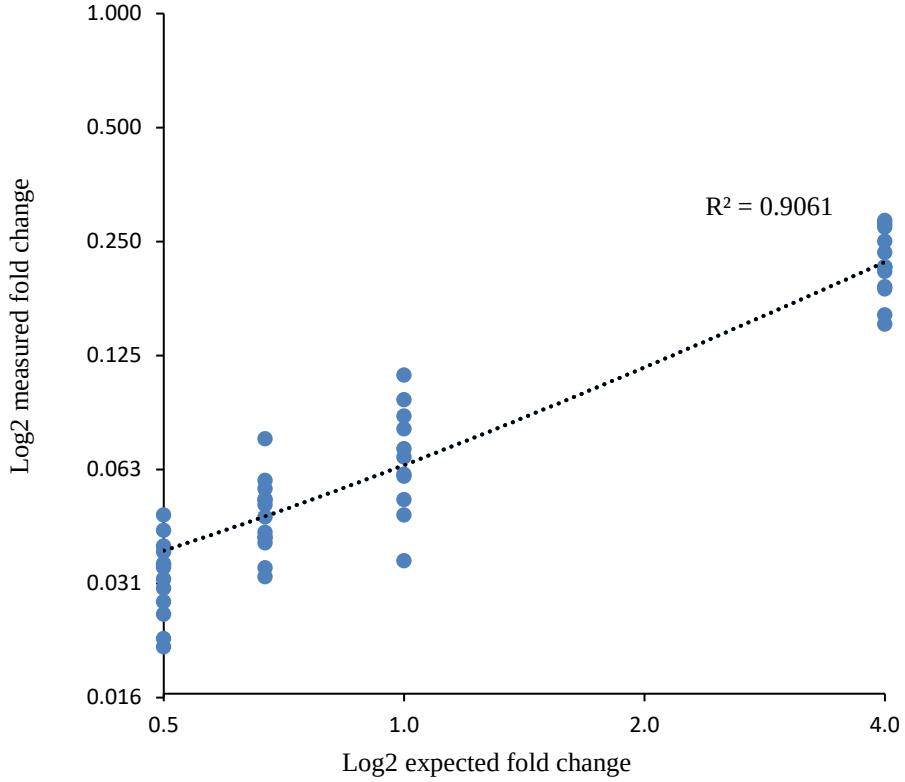

Figure 6.3 Comparison of fold changes for ERCC transcripts. Values were calculated for each transcript from mean fpkm values for all libraries. To achieve an R^2 value > 0.9, transcripts with fpkm values below 0.3 were removed.

6.3.2 Differential gene expression in fetal ovaries

Six libraries, each representing one fetal ovary were compared for day 55 maintenance and restricted groups, and the day 75 maintenance group. Five libraries were available for comparisons in the day 75 restricted group.

6.3.2.1 Overall differential gene expression

A summary of the genes differentially expressed between the ovaries from maintenance and restricted fetuses at both days 55 and 75 is presented in Table 6.1 and Figure 6.4. Complete lists of differentially expressed genes, Ensembl gene id (ENSOARG numbers), expression levels, fold changes, and significance levels are located in Appendix H for day 55 genes and Appendix I for day 75 genes. For all differentially expressed genes, at least one group had an fpkm value > 1.

Cuffdiff analysis reported 69 differentially expressed sequences at day 55. Of these, 34 were down-regulated in restricted animals and 35 were up-regulated in restricted animals. Of the 69 transcripts, 14 assembled contigs with genomic alignment remain unassigned to a gene. This left, by coincidence, 55 assembled contigs at day 55 which aligned to annotated genes. At day 75, 145 sequences were differentially expressed. Of these, 49 were down-regulated in restricted animals and 96 were up-regulated in restricted animals. Of these 145 transcripts, 23 assembled contigs with genomic alignment remain unassigned to a gene. Figure 6.4 presents the gene expression data as volcano plots for day 55 (A) and day 75 (B). Comparison of the two plots show fewer genes are differentially expressed at day 55, but larger gene expression fold changes are evident at this age between maintenance and restricted groups when compared to day 75.

Table 6.1 Summary of differentially expressed genes at days 55 and 75

Differentially expressed sequences	Day 55	Day 75
Total	69	145
Unassigned to a gene identifier	14	23
Known genes common to both ages	16	
Known genes unique to each age	39	106
% up-regulated in restricted group	51%	66%

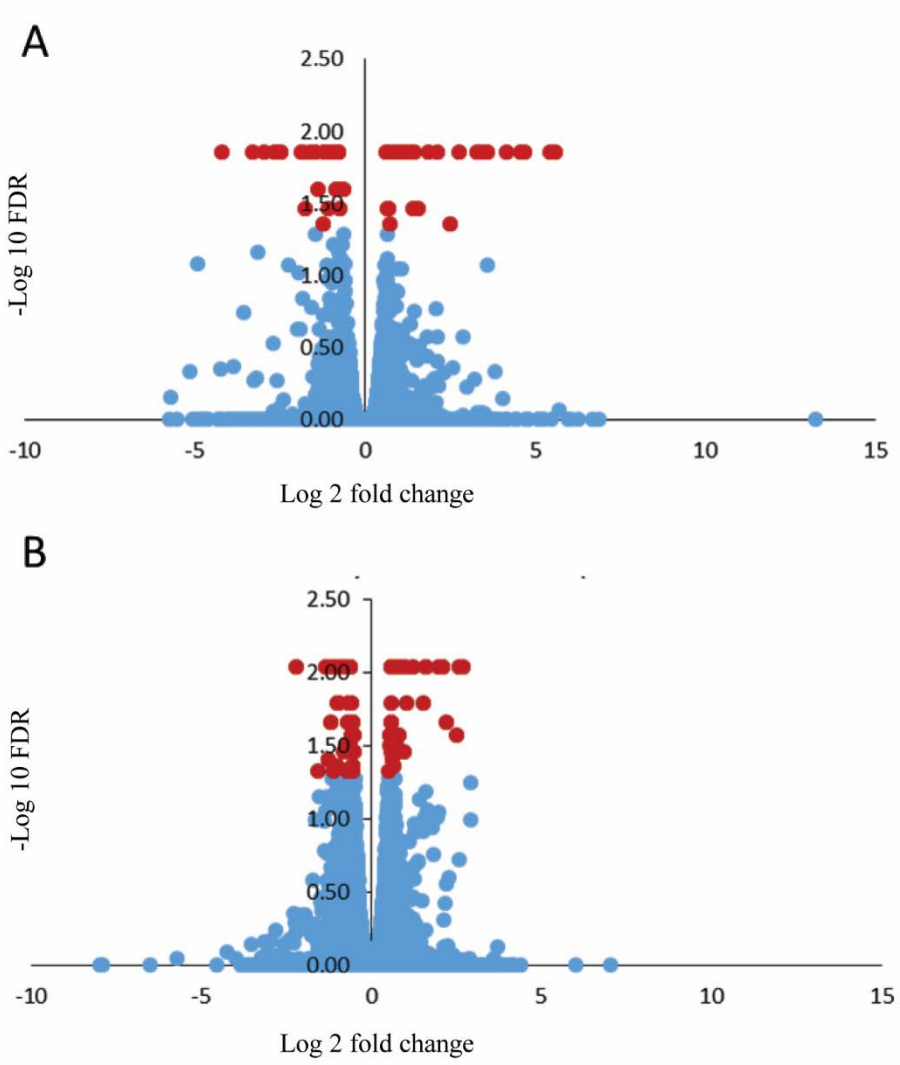

Figure 6.4 Volcano plots illustrating fold change vs FDR (q value). (A) Day 55 genes, **(B)** Day 75 genes. Differentially expressed genes are represented by red dots and genes not differentially expressed are represented by blue dots.

Kilberg and colleagues (*291*) describe the classical mechanisms regulating gene expression in response to nutrient deprivation as being chromatin structure, transcription initiation, RNA splicing, nuclear export of mRNA, and mRNA stabilisation. Many of the differentially expressed genes are involved in these categories (Table 6.2) illustrating at least some predictability to the altered pattern of gene expression. RNA binding proteins have been shown to play a role in RNA stabilisation (*323*).

Table 6.2 Differentially expressed genes fitting classical nutrition response categories

GO annotation	Gene
Chromatin remodelling	*TADA2A, HIST2H2BF*
Transcription	*FIGLA, TADA2A, FOXR1*
RNA splicing or nuclear export	*RBM20*
mRNA stabilisation	*FUS, PNLDC1, DHX40, TDRD15, TDRD10, ZNF19, WDR87, RPS27L, ARMc2, MAEL2, OOEP, TDRKH, TFB1M, DHX58, RPL13, RPL31*

Differentially expressed genes have been assigned to the four categories highlighted by Kilberg and colleagues (*291*) as being responsive to nutrient deprivation.

6.3.2.2 Differential gene expression at day 55

The most represented canonical pathways affected by nutritional restriction at day 55 (Table 6.3) fall into three categories: lipid homeostasis, the citrulline-nitric oxide-Ca^{2+} relationship, and environmental/DNA damage signalling. Lipids are critical to the production of steroids which play an essential role in germ cell development and follicle formation (*146, 270, 324*). Both citrulline and nitric oxide are products of Ca^{2+} dependent arginine metabolism. Citrulline is involved in post-translational protein modification and therefore has the potential to affect many cellular and organ processes (*325*). Nitric oxide and Ca^{2+} are also involved in a wide range of cellular functions. Acute phase response signalling and GADD54 signalling are pathways responsive to environmental changes such as nutrition (*326*).

Table 6.3 Canonical pathways at day 55 affected by gestational nutrition

Pathway	Activation	Function/significance
LXR/RXR activation	downregulated	Nuclear receptors involved in lipid homeostatsis
FXR/RXR activation	napa	Nuclear receptors involved in lipid homeostatsis
Acute phase response signalling	napa	Signalling responses to a variety of stressors including under nutrition. Many of the negative acute phase response proteins bind hormones such as cortisol and retinol
Biotin-carboxyl carrier protein assembly	napa	Involved in fatty acid and lipid biosynthesis
Protein citrullination	napa	Dependent on the pathway involved citrulline production is a Ca^{2+} dependent process
Citrulline-nitric oxide cycle	napa	Citrulline and nitric oxide are products of arginine (process can be reversible)
nNOS signalling in skeletal muscle	napa	Control of blood vessel dilation and muscle contraction
Super pathway of citrulline metabolism	napa	Citrulline is involved in post translational protein modification
Calcium transport 1	napa	Intracellular Ca2+ transport, involved in cell signalling, vitamin and mineral metabolism
GADD54 signalling	napa	GADD45 signalling is in response to environmental stressors (e.g. under-nutrition)
DNA damage induced 14-3-3σ signalling	napa	Controls cell cycle checkpoints in response to DNA damage

napa = no activity pattern available. Napa indicates that IPA cannot determine whether a pathway is activated or inhibited.

Of the 17 most represented functional pathways affected by nutritional restriction at day 55 (Table 6.4), 6 relate to lipid and carbohydrate metabolism, or ion transport/homeostasis. Three relate to inflammatory response, and 4 relate to reproductive ovarian development. The majority of differentially expressed genes involved in the four pathways related to reproductive/ovarian development have been shown to be expressed specifically or preferentially in oocytes. These genes are *ALB, GDF9, FIGLA, NOS1, TMED2,* and *ZP3* (Tables 6.4 and 6.5).

The prominence of germ cell development is further reinforced when IPA overlaps the canonical pathways as a network. Five pathways relating to stem cell differentiation or pluripotency are highlighted as being affected by restricted gestational nutrition (Figure 6.5). These pathways are impacted by the down-regulation of two germ cell specific transcription factors *NANOG* (-2.4 fold) and *POU5F1* (-1.9 fold).

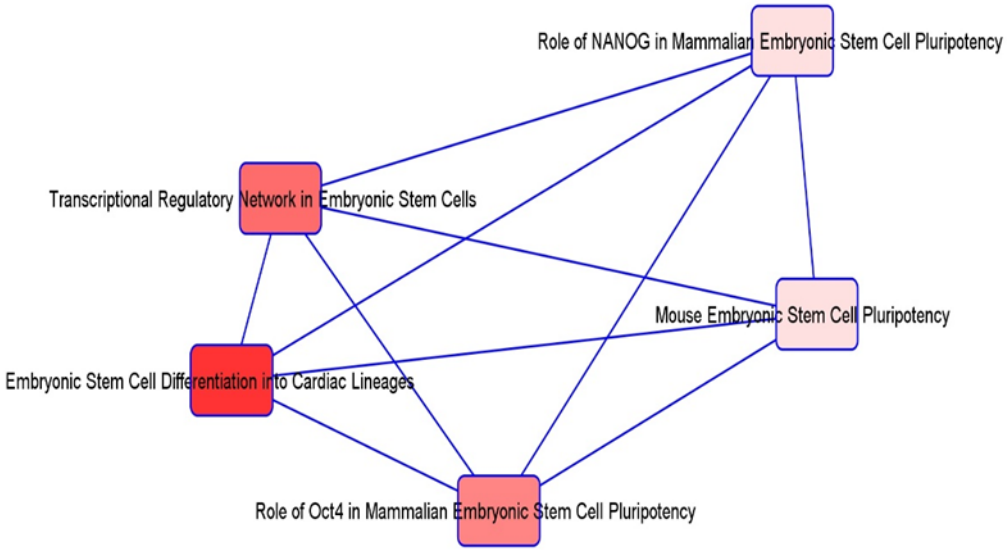

Figure 6.5 Network diagram illustrating stem cell pathways affected. Colour intensity indicates the degree to which the pathway has been affected, based on the number differentially expressed genes involved, their fold change, and q values.

Table 6.4 Functional pathways at day 55 affected by gestational nutrition

Diseases or Functions Annotation	p-value	Activation score	Differentially expressed genes involved in pathway
Quantity of oocytes	4.04E-06	1.980	***FIGLA, GDF9, NOS1, ZP3***
Activation of macrophages	5.85E-03	-1.974	*AGT, APOH, GC, VTN*
Inflammation of body region	1.07E-02	1.844	*AGT, **ALB**, AMBP, APOH, CA5A, FUS, GC, HPX, TNNI3*
Transport of molecules	1.13E-02	-1.652	*AGT, **ALB**, APOH, ATP2C2, FGB, FUS, GC, **GDF9**, HPX, **NOS1, TMED2, ZP3***
Inflammatory response	5.04E-03	-1.424	*AGT, AHSG, APOH, GC, **NOS1**, SERPINA1, VTN, **ZP3***
Conversion of lipid	9.31E-04	1.413	***ALB**, AMBP, HPX, **NOS1***
Binding of cells	4.61E-03	-1.407	*AGT, APOH, FETUB, SERPINA5,VTN, **ZP3***
Fibrosis	8.56E-03	-1.297	*AGT, **ALB,** HPX, **NOS1**, TNNI3, VTN*
Development of reproductive system	3.73E-03	1.199	*AHSG, **ALB**, DNAH9, **FIGLA, GDF9**, SERPINA5, **TMED2, ZP3***
Gonadogenesis	1.71E-03	1.199	*AHSG, **ALB**, DNAH9, **FIGLA, GDF9,** SERPINA5, **ZP3***
Quantity of carbohydrate	1.50E-03	-1.154	*ACACB, ACVR2B, AGT, **ALB,** AMBP, CA5A, **NOS1***
Mass of gonad	4.04E-03	1.067	*AGT, **GDF9, NOS1, ZP3***
Activation of cells	6.37E-03	-1.053	*AFP, AGT, **ALB**, APOH, ERC2, GC, SERPINA1, VTN, **ZP3***
Flux of Ca2+	2.29E-04	-1.026	*AGT, **ALB**, ATP2C2, ERC2, GC, VTN*
Cellular homeostasis	7.20E-03	-1.031	*ACACB, AGT, **ALB**, ATP2C2, ERC2, GC, **GDF9**, HPX, **NOS1,** SERPINA1, TNNI3, VTN*
Ion homeostasis of cells	2.45E-03	-1.026	*AGT, **ALB**, ATP2C2, ERC2, GC, TNNI3, VTN*
Cell movement	7.01E-03	-1.006	*AFP, AGT, AHSG, **ALB**, CDK1, CHL1, DNAH1, FGB, GC, **GDF9, NOS1**, SERPINA1, SERPINA5, VTN, **ZP3***

Pathways are ranked by activation score (Section 6.2.2). Genes in bold are expressed either specifically or preferentially in germ cells, referenced from, *ALB* (*327*), *GDF9* (*327*), *NOS1*(*328*), *TMED2* (*329*), *ZP3* (*327*), and *FIGLA* (*330*).

Extensive examination of the literature revealed that of the 55 known genes identified at day 55 as differentially expressed by Cuffdiff, the cell specific expression pattern of 35 genes has been published. Of these 35 genes, 13 are expressed in all ovarian cell types including germ cells, and 16 are expressed preferentially, or solely in germ cells (Figure 6.6 and Table 6.5). Four of the genes are reported to be expressed specifically in adult oocytes, and only 2 have been shown not to be expressed in germ cells. Of these 55 genes, 20 with an unknown expression pattern are involved in functions such as reproductive development, gonadogenesis, as well as basic cellular functions including homeostasis, and ion/molecule transport. It seems likely that some of these 20 genes are expressed in germ cells. Collectively, this data is consistent with a marked effect on germ cell gene expression at day 55 by the nutritional restriction regime used in this study.

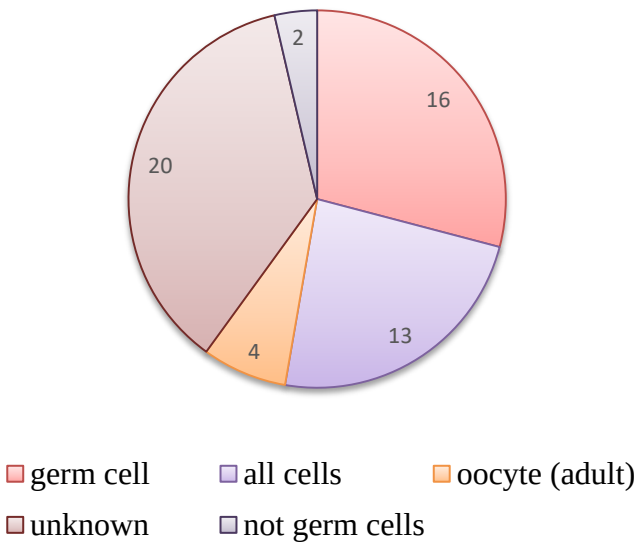

Figure 6.6 Cell specific expression pattern of differentially expressed known genes at day 55 of gestation. Segment size represents proportion of the 55 differentially expressed genes. Numbers refer to number of genes from a total of 55 differentially expressed genes.

Table 6.5 Cell specific expression pattern of differentially expressed genes at day 55

Cell specific expression	Genes
Germ cell specific or preferential (fetal) Total = 16	*ALB(329), CDC2(331), FETUB(332), FIGLA(330), FOXR1(330), GDF9(327), HSP70 (I and II)(333, 334), NEFH, NOS1(328), PADI6, PNLDC1(330), RIBC1, TADA2A, TMED2(329), ZP3(327)*
Oocyte specific or preferential (adult) Total = 4	*ACAB, ATP2C2(335), SERPINA1(333), WDR87(335)*
Expressed in all cells Total = 13	*ACVR2B(121), AGT(320), BAIAP2L1(320), CHL1, DHX40, FAM3C, FUS, GC, KIAA1324L, RPS27L, SERPINA5, SPCS3, TNNI3*
Minimal germ cell expression Total = 2	*SH2D4A, VTN**
Expression pattern unknown Total = 20	*AFP, AHSG, AMBP, APOH, C1orf112, CA5A, CALY, CRTAC1, DNAH1, DNAH9, ERC2, FGB, HIST2H2BF, HPX, ITIH2, SAMD15, SPESP1, SPP2, TDRD15, ZNF19*

Unless indicated, cell specific expression is based on Jamieson and colleagues (*320*). * Expression is based on unpublished IHC data from Santa Cruz Biotechnology Inc., Dallas, TX, USA.

The importance of germ cell specific gene expression is illustrated when the ten most up-regulated genes at day 55 are examined (Table 6.6). Of these 10 genes, 7 are known to be specifically or preferentially expressed in germ cells. The cell specific expression of the remaining three is unknown in fetal ovaries. The GO annotations of these ten genes can largely be assigned to two categories: nucleic acid binding, and ion transport. Of the exceptions to this, *SAMD15* has no assigned functional annotation, and the role of GDF9 in fetal ovaries has yet to be clearly established (*336*).

Table 6.6 Ten most up-regulated genes at day 55 of gestation in response to nutritional restriction

Gene	Reported expression	Maintenance fpkm	Fold change	GO annotation(s)
ZP3	germ cell	0.1	58.4	ion transport
FOXR1	germ cell	0.3	18.3	transcription factor, nucleic acid binding
FIGLA	germ cell	0.8	9.8	transcription factor, nucleic acid binding
PADI6	germ cell	2.5	6.4	ion transport
KIAA1324L	unknown	0.5	6.0	ion transport
HIST2H2BF	unknown	5.3	5.6	DNA binding
GDF9	germ cell	2.5	3.7	cytokine activity
NOS1	germ cell	0.4	3.4	ion transport, protein binding
PNLDC1	germ cell	2.5	3.1	nucleic acid binding
SAMD15	unknown	2.4	2.9	unknown function

While examination of the up-regulated genes highlights the importance of germ cell gene expression, examination of down regulated genes illustrates that other cells and mechanisms may be affected by the nutrition regime. Of these ten genes (Table 6.7), only two, *FETUB* and *ALB*, have been shown to be specifically or preferentially expressed in germ cells. The ten most down-regulated genes can be assigned to only two GO categories: carrier proteins of the albumin family, and those with protease inhibitor activity (Table 6.7).

Table 6.7 Ten most down-regulated genes at day 55 of gestation in response to nutritional restriction

Gene	Expression	Maintenance fpkm	Fold change	GO annotation(s)
AFP	unknown	30.0	-48.2	carrier protein albumin family
FETUB	germ cell	10.6	-43.6	protease inhibitor
SERPINA1	unknown	84.8	-25.5	protease inhibitor
AMBP	unknown	7.2	-25.2	protease inhibitor vesicle development
GC	all	5.3	-24.0	carrier protein albumin family
AHSG	unknown	184.8	-17.7	protease inhibitor vesicle development
ALB	germ cell	16.1	-12.0	carrier protein albumin family
VTN	not germ cell	5.6	-11.6	binds to protease inhibitors
SPP2	unknown	6.4	-10.4	protease inhibitor
ITIH2	unknown	2.2	-10.2	protease inhibitor

6.3.2.3 Differential gene expression at day 75

Based on stereological analysis, fetal ovaries exposed to the restricted nutrition regime had a 1.5 fold increase in germ cell number (Section 4.3.2). As the gene expression data represents the whole ovary, this fact must be kept in mind when interpreting the data, i.e. a 1.5 fold increase in expression of germ cell specific genes is likely to reflect the increase in germ cell number, and not an increased expression in each germ cell. The published cell specific expression of genes identified as being differentially expressed by Cuffdiff in fetal ovaries at day 75 is presented in Figure 6.7 and Table 6.8.

Of the 122 differentially expressed genes at day 75, 25 are known to be specifically or preferentially expressed in germ cells, 11 are known to be specifically or preferentially expressed in adult oocytes, and 34 are known to be expressed in all cells of the fetal ovary including germ cells. The ovarian cell specific expression of 39 genes is unknown and 13 are known to either not be expressed in germ cells or have low expression in germ cells.

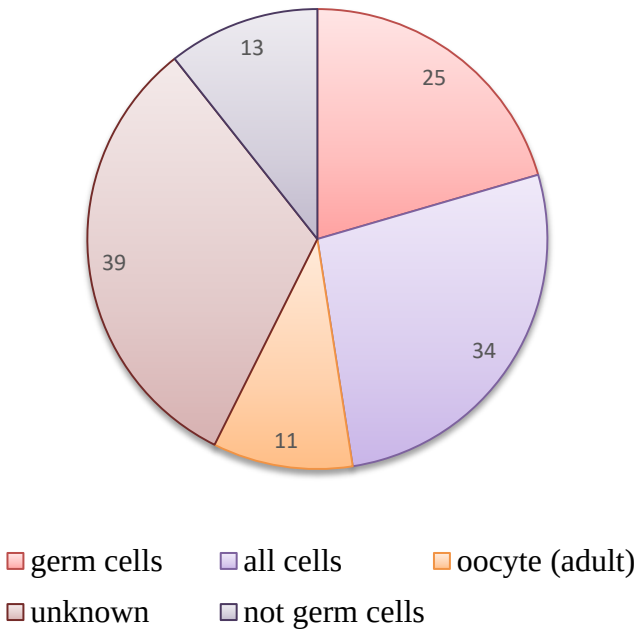

Figure 6.7 Cell specific expression pattern of differentially expressed known genes at day 75 of gestation. Total of 122 genes. Segment size represents proportion of the 122 differentially expressed genes. Numbers refer to number of genes.

Table 6.8 Cell specific expression pattern of differentially expressed genes at day 75

Cell specific expression	Genes
Germ cell specific or preferential (fetal) Total = 25	*ALB(329), CCDC64, CLIP4, COCH, FIGLA(330), FOXR1(330), LHX8(337), LIG1(330), MAEL(338), MAP3K15(330), MATER(338), MVP(334), NETO2, NOBOX(332), NOS1(339), OOEP(340), PADI6, PNLDC1(330), RIBC1, TDRKH(330),TFB1M, TMED2(329), YBX2(330), ZNF541(330), ZP3(327)*
Oocyte specific or preferential (adult) Total = 11	*ADAMTS14(335), ARMC2(335), ASB11(335), C11orf16(335), CHL1(335), HIP1R(335), PLG(335), SERPINA1, SLC12A3(335), TTN(335), WDR87(335)*
Expressed in all cells Total = 34	*ALDH1A1, APAF-1, BAIAP2L1, CCDC88C, CEP164, COX5A, DOCK5, ENTPD4, FSTL4, GLRX, ITM2B, JAG1, JAK3(320), KCTD9, LGMN, MYBPC3, NLRC5, PARP2, PCBD1, PGS1, PHTF1, POLDIP2, ROR2, RP9, RPL13, RPL31,SEMA3G, SLC25A15, SLC7A6, SPON1, TMC4, UBE2Q1, UNC13C, UPF3A*
Minimal germ cell expression Total = 13	*CALB2(341), DACH1, FAP, GPRC5B, GREB1, HIPCAL1, HSPG2, PLXNC1, PTN, RALB, SLC2A1, TKT, GUCY1A3*
Expression pattern Unknown Total = 39	*ACAB, AFP, AHSG, AMBP, APCDD1L, C20orf27, C7orf25, CADPS2, CCL16, CHRDL1, CNR1, CNTNAP4, DHX58, DLEC1, DNAH1, EPB41L4B, FAM204A, GPANK1, HAX1, HSPA6, IRF8, ISG17, MAP7, MOXD1, MX1, MYH15, PRELP, RBM20, RYR3, SCD5, SCN1A, SLC12A1, SLC5A6, SLC8B1, SPTBN5, TDRD10, TDRD15, TSKS, WIF1*

Unless specified, cell specific expression is based on Jamieson and colleagues (*320*).

The pattern of differential gene expression at day 75 becomes clearer when the most up-regulated and down-regulated genes (based on fold changes) are examined. Of the top ten up-regulated genes (Table 6.9), 3 are known to be specifically or preferentially expressed in germ cells. One is expressed in all cells including germ cells, while the expression pattern of the remaining 6 in the fetal ovary is unknown (3 of these 6 are known to be expressed specifically or preferentially in adult oocytes).

The ten most down-regulated genes at day 75 (Table 6.10) bear a similarity to those down-regulated genes at day 55, with protease inhibitors and carrier proteins of the albumin family featuring prominently. The difference in fold change between the two groups at day 75 is much less than that observed at day 55, with the exception to this being *CCL16*. This gene was expressed in maintenance ovaries but no expression was evident in restricted ovaries. Therefore the fold change reported as -100 is an artificial value (Section 6.2.2). The average fold change of the ten most down-regulated genes at day 55 was -25.5 while at day 75 the average fold change (excluding *CCL16*) was -4.2

Table 6.9 Ten most up-regulated genes at day 75 of gestation in response to nutritional restriction

Gene	Expression	Maintenance fpkm	Fold change	GO annotation(s)
SLC12A3	oocyte (adult)	1.2	2.94	ion transport
ADAMTS14	oocyte (adult)	5.3	2.52	peptidase activity
TDRD10	germ cell	21.8	2.29	nucleic acid binding
MATER	germ cell	3.1	2.29	germ cell development
RBM20	unknown	1.7	2.27	nucleic acid binding
CHL1	oocyte (adult)	9.7	2.25	protease binding
SPTBN5	unknown	1.1	2.19	actin binding
NLRC5	all cells	2.2	2.17	DNA binding
TSKS	germ cell	4.3	2.17	protein kinase binding
RYR3	unknown	0.9	2.16	ion transport

Table 6.10 Ten most down-regulated genes at day 75 of gestation in response to nutritional restriction

Gene	Expression	Maintenance fpkm	Fold change	GO annotation(s)
CCL16	unknown	2.3	-100	chemokine activity
AMBP	unknown	9.0	-6.5	protease inhibitor
AHSG	unknown	108.9	-5.1	protease inhibitor
PLG	unknown	1.8	-4.7	protease inhibitor
SERPINA1	oocyte (adult)	57.4	-4.3	protease inhibitor
AFP	unknown	12.5	-4.20	carrier protein albumin family
ALB	unknown	16.3	-4.01	carrier protein albumin family
HIP1R	oocyte (adult)	52.6	-3.84	vesicle mediated transport
APCDD1L	unknown	21	-3.01	membrane component
MOXD1	unknown	15.7	-2.33	ion transport

The top canonical pathways identified by IPA analysis as being affected by restricted gestational nutrition at day 75 are presented in Table 6.11. IPA predicts down-regulation of both the LXR/RXR pathway as well as production of nitric oxide and reactive oxygen species in macrophages.

In addition to LXR/RXR, the FXR/LXR pathway is also identified as being affected. The differentially expressed genes identified in these pathways are the same as those at day 55 (*AHSG*, *ALB*, *AMBP* and *SERPINA1*). The analysis identifies the availability of HDL (high density lipoproteins) as being critical for these pathways. A relationship between vitamin C (ascorbate) pathways, nitric oxide, and protein citrullination (all identified as affected pathways by IPA) is also known, with ascorbate reported as inducing production of nitric oxide (*342, 343*).

Functional pathways identified by IPA as being affected at day 75 of gestation are presented in Table 6.12. Of the 10 functional pathways, 7 relate to gonadal development, with germ cell specific/preferential genes being key to these effects. Based on the gene expression data, IPA assigns a z score > 2 to both quantity of oocytes and quantity of cells, predicting up-regulation

of these two functional pathways. This result is consistent with the increase in germ cell numbers observed by stereology at this age. While quantity of protein in blood is listed as being affected, this is based on a global analysis (not ovary specific) and assumes that the observed gene expression patterns are similar in most tissues. It is of interest to note that the cell death pathway is listed as affected, although the z score of 1 is relatively low (a score of > 2 or < 2 being required for IPA to predict up or down regulation of function).

Table 6.11 Canonical pathways at day 75 affected by gestational nutrition

Pathway	Activation	Function/significance
LXR/RXR activation	down-regulated	Nuclear receptors involved in lipid homeostasis
Production of nitric oxide and reactive oxygen species in macrophages	down-regulated	Immune response, infection control
Acute Phase response signalling	neutral	Signalling responses to a variety of stressors. May indicate response to post restriction compensatory growth
FXR/RXR activation	napa	Nuclear receptors involved in lipid homeostasis
Vitamin C transport	napa	Involved in growth, proliferation and development
Ascorbate recycling	napa	Antioxidant activity and vitamin C metabolism.
Glutathione redox reactions	napa	Involved in controlling ROS
Biotin-Carboxyl carrier protein assembly	napa	Fatty acid and lipid biosynthesis
Tyrosine biosynthesis	napa	Synthesis from phenylalanine. Involved in cell to cell signalling
Nitric oxide signalling in skeletal muscle	napa	Cardiovascular signalling. Key regulator is Ca^{2+}
Citrulline nitric oxide cycle	napa	Citrulline and NO are products of arginine (process can be reversible).
Protein citrullination *	napa	Post translational protein modification

napa = no activity pattern available. * indicates genes associated with this pathway were oocyte specific/preferential.

Table 6.12 Functional pathways at day 75 of gestation affected by gestational nutrition

Diseases or Functions Annotation	p-value	Activation score	Differentially expressed genes involved in pathway
Quantity of oocytes	4.67E-06	2.219	*FIGLA, NOBOX, NOS1, YBX2, ZP3*
Quantity of cells	2.05E-03	2.157	*AMBP, CADPS2, DACH1, FIGLA, ZP3, GUCY1A3, HAX1, HSPG2, IRF8, JAG1, JAK3, LGMN, LHX8, LIG1, MAP7, MYBPC3, NLRC5, NOBOX, NOS1, PLG, PTN, ROR2, SERPINA1, SLC2A1, YBX2*
Quantity of germ cells	1.03E-04	1.969	*FIGLA, MAP7, NOBOX, NOS1, ROR2, YBX2, ZP3*
Quantity of protein in blood	1.17E-03	-1.305	*ACACB, AHSG, CNR1, DHX58, LGMN, NOS1, PCBD1, PLG, PTN, SLC12A1, UBE2Q1*
Gonadogenesis	5.00E-04	1.154	*AHSG, ALB, FIGLA, LHX8, MAEL, MAP7, NOBOX, PLG, PTN, TDRKH, YBX2, ZP3*
Development of internal genitalia	8.72E-06	1.091	*AHSG, ALB, FIGLA, LHX8, MAP7, NOBOX, PLG, PTN, YBX2, ZP3*
Development of ovary	4.43E-05	1.091	*ALB, FIGLA, LHX8, NOBOX, PLG, YBX2, ZP3*
Folliculogenesis	1.15E-04	1.091	*ALB, FIGLA, NOBOX, PLG, YBX2, ZP3*
Adipogenesis	9.75E-03	-1.067	*ALDH1A1, CNR1, PLG, ROR2*
Cell death	1.94E-04	1.007	*AFP, ALB, ALDH1A1, CADPS2, CHL1, CNR1, COX5A, DACH1, DHX58, FAP, FIGLA, GLRX, HAX1, HIP1R, HSPG2, IRF8, ITM2B, JAG1, JAK3, LGMN, LIG1, MAEL, MVP, MX1, MYBPC3, NOS1, PARP2, PLG, PTN, RALB, ROR2, RPL13, RPL31, RYR3, SCN1A, SERPINA1, SLC2A1, TFB1M, TTN, WIF1, YBX2*

6.3.3 Validation of RNAseq using Nanostring

A comparison of fold changes derived from RNAseq and Nanostring is illustrated in Figure 6.18 and Table 6.13. A good overall correlation between fold changes observed using Nanostring and RNAseq was achieved. Figure 6.8 presents the correlation between Nanostring and RNAseq results as a Bland Altman plot. Bland Altman analysis plots the mean of two values being compared against the difference between the two means, and is extensively used to compare agreement between two different techniques (*344*). The comparison shows good correlation between the two techniques with an R^2 value of 0.8986. Some divergence in the magnitude of the fold changes, but not the direction, was noted. This arose where values for individual genes were below the detection limit of the Nanostring assay (Table 6.13) in a particular fetal ovary RNA sample. Where this occurred more than once over the 12 day 55 samples, or the 11 day 55 samples, the gene was removed from the comparative analysis.

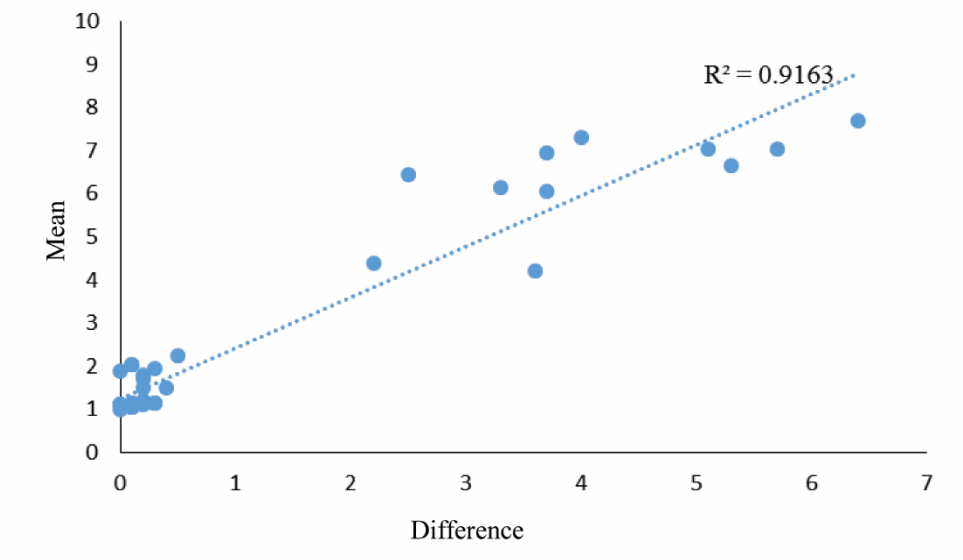

Figure 6.8 Bland Altman plot comparing fold changes recorded for Nanostring and RNAseq.

Table 6.13 Comparison of Nanostring and RNAseq fold changes

	Day 55		Day 75	
	RNAseq fold change	**Nanostring fold change**	**RNAseq fold change**	**Nanostring fold change**
HSD3B	1.0	1.0	1.1	1.1
ACVR2B	-1.1	-1.1	1.2	1.1
AFP	-48.2	-12.2*	-4.2*	-9.9
AGT	-6.6	-18.7*	-3.2*	-9
AHSG	-17.6	-11.4	-5.1	-8.8
AHR	1.2	1.2	1.1	1.2
ALB	-12.0	-10.6	-4	-9.3
ALDH1A1	1.1	1.3	-1.7	-1.9
AMBP	-25.5	-11.5*	-5.3	-9.3
APOA2	-8.0	-13.7	-5.2	-7.7
APOC3	-13.9	nd	-7.2	-10.2*
APOH	-4.0	-5.9	-2.4	-6.0
BMPR2	1	1	1.1	1.0
CCNB1	-1	1.1	1.2	1.0
CRABP2	-1.3	-1.2	1.3	1.1
CYP11A1	1.1	1.1	1.1	1.0
CYP17A1	-1.9	-1	1.3	1.0
DACH1	1	1	-1.1	-1.2
DAZL	-1.0	-1.1	1.0	1.1
FETUB	-43.4	-16.4*	-4.5	-7.8
FIGLA	9.8	12.6*	2.0	2.5
FSTL4	-1.0	-1.1	1.9	1.9
GC	-23.9	-19.9*	-4.5*	-9.6
GDF9	3.7	3.7*	1.6	1.8
GRIA1	-1.1	-1.7*	1.0	-1.3
INHBA	-1.2	-1.1	1.1	1.0
ITIH2	-10.2	nd	-3.3	-5.5
LDLR	-1.2	1.1	-1.2	-1.1
LHR	-1.2	-1.3	1.1	-1.1
MGST1	-1.0	1.0	1.0	1.0
NOBOX	14.4	nd	2.1	1.8
NOS1	3.4	14.3*	2.0	2.1
PLAC1	1.2	1.0	1.3	1.0
RARA	-1.1	1.0	1.1	1.1
RARES2	-1.2	1.0	1.1	1.1
SERPINA1	-25.5	-10.3	-4.2	-7.9
SPP2	-10.4	-20.5*	-4.5	-10.9*
STAR	1.1	1.3	1.1	1.0
ZP3	58.4	nd	2.0	2.1
ZP2	6.2	39.3*	1.3	1.7
ZP4	23.4	14*	1.4	1.6

* indicates one or more values are below the detection limit of the Nanostring assay. *PADI6, ILO-15L, INSL3* and *FSHR* were undetectable using Nanostring at both gestational ages.

6.4 Discussion

The primary aim of RNAseq was to identify changes in the expression of genes or pathways in the fetal ovary following restricted gestational nutrition. Significant changes to the ovarian transcriptome are apparent at the completion of restricted nutrition at day 55.

Key findings from the gene expression analysis include

- up-regulation of key germ cell genes associated with nucleic acid binding and ion transport at day 55
- down-regulation of specific carrier protein genes and protease inhibitors at day 55
- higher ovarian expression of a number of germ cell genes at day 75 reflective of the increased germ cell number at this age
- continued down regulation of protease inhibitors and carrier protein genes at day 75 although with reduced fold changes than those observed at day 55

At day 55 of gestation, changes in gene expression were evident before morphological changes to ovarian or germ cell development were observed by histology. Some of these changes in gene expression are still evident at day 75, 20 days following the change to an ad libitum diet for all animals, although the fold changes observed are considerably less than those at day 55. The largest fold change observed in up-regulated genes at day 75 was a 2.9 fold increase in the ion transport gene *SLC12A3*. Given that many of these genes are germ cell specific or preferential in their expression pattern, this suggests that the observed up-regulation of many of these genes reflects the increased number of germ cells present in the fetal ovaries from fetuses exposed to the restricted nutrition regime. Thus, while the ovary as a whole may be expressing more of these genes, it is likely individual germ cells are not. This pattern of fold change reflecting the increased number of germ cells is also exhibited by many other germ cell genes not reported as being significantly differentially expressed between groups, for example, *GDF9* shows a 1.5 fold increase, *POU5F1* a 1.5 fold increase, and *SPO11* a 1.2 fold increase.

These observations are consistent with changes to the transcriptome in restricted animals being temporal, allowing normal ovarian development in a reduced nutrition environment. Following the change to an ad libitum diet, the transcriptome gradually returns to a state consistent with ad libitum nutrition. This interpretation of the data is consistent with the hypothesis developed from Chapter 4 which examined morphological development of the fetal ovary. This suggests the change from restricted to ad libitum nutrition, while the transcriptome briefly remains

attuned for restricted nutrition, is the critical factor in establishing the observed alterations to ovarian development.

To understand how these reported differentially expressed genes may affect ovarian or germ cell development, a number of factors need to be considered. RNAseq has reported differential gene expression, however this may not necessarily translate into increased or decreased levels of corresponding proteins. Even assuming that protein levels are correlated to mRNA expression levels, post translational protein modifications have not been considered. Given that citrullination is one pathway affected, and that citrulline is involved in post-translational protein modification, it may affect cellular processes independent of differences in gene expression. Differences in alternative splicing of genes and consequently differences in isoforms of proteins have also not been considered in the analysis thus far. Despite these factors, consideration of the genes and their functions may give insights into possible mechanisms whereby restricted gestational nutrition may affect ovarian development. Given that the number of differentially expressed genes reported is relatively low (69 at day 55 and 145 at day 75), the implication is that a specific, targeted mechanism may be involved rather than a broad based affect.

Of the genes up-regulated at day 55, three are known transcription factors (*FIGLA, TADA2A* and *FOXR1*). *FIGLA* in particular is known to regulate expression of the zona pellucida genes (*345*). The analysis reported a significant 58 fold up-regulation of *ZP3* at day 55. It is also interesting to note that while not statistically significant, the two other zona pellucida genes expressed at this stage were also up-regulated. A 6 fold change was reported for *ZP2*, while a 45 fold change was reported for *ZP4*. Electron microscope examination of sheep fetal ovaries at day 55 of gestation does not reveal any indications of zona pellucida formation (*34*). However, similar to the results from this study, expression of *ZP3* has been observed in human ovaries prior to follicle formation (*34, 346*). GO annotations for *ZP3* include both signal transducer activity and Ca^{2+} channel activity. Based on this evidence, it appears likely that at this stage of development rather than zona pellucida formation, ZP3 is playing some alternative role in germ cell development. The role of ZP3 in Ca^{2+} channel activity is of particular interest. IPA identified ion transport as an affected pathway, and there are at least three other up-regulated germ cell specific genes involved in ion transport: *NOS1, PADI6* and *KIAA1324L*.

The importance of Ca^{2+} availability in the germ cells leads into, arguably, the affected pathways with potentially the most consequences for development. These pathways are those relating to citrullination, Ca^{2+}, and nitric oxide production by *NOS1*, with *NOS1* expression reportedly oocyte specific or preferential (*328*). Nitric oxide is known to be involved in Ca^{2+} homeostasis

at a number of different levels (*347*). Additionally, conversion of arginine to nitric oxide and citrulline by *NOS1* and *PADI6,* is a Ca^{2+} dependent reaction (*348*). This reaction links all three pathways (citrullination, Ca^{2+}, and nitric oxide production by *NOS1*) indicated by IPA as being affected by restricted gestational nutrition. The 3.4 fold up-regulation of *NOS1* in restricted ovaries at day 55 may be in response to changes in insulin levels as a result of restricted nutrition. Elevated insulin levels have been shown to increase *NOS1* mRNA levels in cerebral astrocytes and neurons (*349*). This reaction is also catalysed by Ca^{2+} dependent peptidylarginine deaminases such as *PADI6* (*325*), an oocyte specific gene differentially expressed at both gestational ages.

Interestingly, three pathways (vitamin C transport, ascorbate recycling, and glutathione redox reactions) identified by IPA as being affected by restricted gestational nutrition at day 75 are involved in vitamin C metabolism or transport. Vitamin C (ascorbate) is one compound that can potentiate nitric oxide synthesis (*343*). The importance of nitric oxide and citrulline in fetal development have been highlighted by Wu and colleagues (*160*). They emphasise the relationship between impaired nitric oxide production and intra-uterine growth retardation (*350*), a relationship that can be reversed by arginine supplementation (*351*). Further, in sheep allantoic fluid, concentrations of citrulline increase 18 fold between days 30 and 60 (*352*). While the emphasis of the Wu review is on placental development, it is notable that maternal nutrition restriction decreases fetal plasma arginine concentrations in the pig at day 60 of gestation (*353*). In sheep, restricted maternal nutrition between days 28 and 78 of gestation decreases maternal and fetal plasma levels of both arginine and citrulline (*354*).

Citrulline is involved in post translational protein modification. Alterations to post translational protein modification has the potential to affect many cellular functions. Determining if this is indeed a factor in these ovaries requires an in-depth proteomics investigation to examine the structure of proteins susceptible to the effects of citrulline and is beyond the scope of this study.

The role of nitric oxide in ovarian development is complex as nitric oxide is involved in numerous cell and developmental processes, additionally *NOS* is present in three distinct isoforms (*348*). *NOS1* was first discovered in neuronal cells and is often referred to as neuronal or brain *NOS*, while *NOS2* or *iNOS* is referred to as inducible *NOS. NOS3* or *eNOS* refers to endothelial cell *NOS*. Up-regulation of *NOS1,* and genes involved in ion transport, particularly Ca^{2+}, suggest that there are increased levels of nitric oxide in the germ cells of nutrition restricted fetuses.

Nitric oxide is known to play a role in DNA damage and repair although its precise role is not clear. While most studies highlight the negative effects of nitric oxide on DNA damage and repair, many of these studies use exogenously administered nitric oxide. However, Phoa and Epe (*355*) conclude that nitric oxide generates DNA damage only inefficiently, and only selectively inhibits the repair of oxidative DNA base modifications. Further, Phoa and Epe state that nitric oxide can protect against some forms of DNA damage (*355*). Patel and colleagues (*356*) showed that nitric oxide is required for cell proliferation once DNA damage is repaired. Zhang reported that fetal germ cells from mice deficient in *NOS3*, and exposed to the environmental oestrogen bisphenol A, showed an increase in chromosomal errors during meiosis (*357*). As *NOS1* is preferentially expressed in the germ cells (*328*), and is up-regulated in restricted fetal ovaries at a time of significant proliferation, apoptosis, and also meiosis, it seems likely that this up-regulation may have an effect on the DNA status in these cells. Given the opposing views in the literature, it is difficult to determine whether this effect is positive or negative. However, the observation that more germ cells are present in day 75 restricted ovaries is consistent with a positive effect rather than a negative effect. Given this evidence, it seems that nitric oxide may be one mechanism by which restricted gestational nutrition may be affecting ovarian development by affording some protection to germ cell DNA or by facilitating DNA repair in these cells.

Nitric oxide is also known to play an important role in angiogenesis, although this function is normally attributed to *NOS3* (*358*), which in the fetal ovary is predominantly expressed in endothelial cells (*320*). While Zhang (*328*) attributes most *NOS1* expression to germ cells, Jamieson and colleagues (*320*) attribute the majority of *NOS1* expression to the supporting or pre-granulosa cells as well as germ cells. The differences between these two studies likely reflects differences in the gestational age of the animals. The differences notwithstanding, given the expression of *NOS1* either adjacent to, or within germ cells, it seems likely that any effect of differential expression will be focused in the germ cells. It seems unlikely that the differences in *NOS1* expression would affect angiogenesis. Expression of *NOS1* is relatively low compared to *NOS3* expression (0.5 fpkm compared to 11.3 fpkm in maintenance fetuses). *NOS3* expression is also concentrated in endothelial cells and therefore likely to control angiogenesis (*348*).

While the up-regulation of germ cell specific genes is a notable pattern in this data set, the down-regulated genes observed are likely to play an important role. The fold changes in these genes are significantly greater than those observed in the up-regulated genes. Specifically,

expression of genes for carrier proteins of the albumin family change significantly with *AFP*, *ALB* and *GC* all showing large negative fold changes. These genes are primarily expressed in liver and bind to a number of compounds including steroids and cations such as Ca^{2+} in plasma (*359*). The expression pattern and role of the members of the albumin family in the fetal ovary has yet to be fully investigated. it is therefore difficult to relate the differences in gene expression to the changes observed in the fetal ovary. However, the characteristic of members of the albumin family to bind Ca^{2+} may signify an indirect involvement in Ca^{2+} dependent nitric oxide synthesis.

AFP is considered the fetal equivalent of albumin. Expression of *AFP* in the fetus declines with gestational age as expression of *ALB* increases. By birth, liver expression of *AFP* is negligible (*360*). Despite its abundance early in fetal life, AFP is not essential for development. It is required for female fertility as *AFP* null mice develop to adulthood, but are infertile (*360*). This infertility is thought to be due to effects on the hypothalamus-pituitary system where the oestrogen binding properties of AFP have a neuro-protective effect on the fetal brain (*361*). At day 55 of gestation, the hypothalamic-pituitary system has yet to develop in sheep (*278*), and therefore it seems unlikely that differences in *AFP* expression at this age are producing an effect at the hypothalamus or pituitary. However, expression of *AFP* has been observed in the ovarian follicles of pregnant rats and the level of this expression appears related to the health of the follicle, suggesting some link between this gene and ovarian function (*362*).

Based on the known binding roles of these albumin like proteins, ovarian expression of these genes may serve to bind and therefore regulate intra-ovarian or intra-cellular levels of compounds that bind to the albumin family (e.g. steroids, Ca^{2+}). Down-regulation of these genes may serve to increase the availability of the unbound compounds for cellular functions, conceivably an important concept given the important roles of steroids in gonadogenesis, and the importance of Ca^{2+} discussed previously.

Of particular interest is the down-regulation of protease inhibitors *FETUB, SERPINA1, SERPINA5, AMBP, AHSG, SPP2, ITIH* at day 55, and *AMBP, AHSG, PLG, SERPINA1* at day 75. Proteases and their inhibitors control a wide range of biological processes including cell signalling, apoptosis, and inflammation. The process can either be destructive (destroying the proteins function) or positive (activation of a function or pathway) (*363*). Arguably, the most relevant proteases to this study are the caspases, as these are critical in germ cell apoptosis.

The role of protease inhibitors in fetal ovaries is unclear. Of the protease inhibitors identified by Cuffdiff with differential expression levels between groups, none are known to be germ cell specific in their expression pattern. Further, the cell specific expression pattern for these protease inhibitors has not been published for fetal ovaries. Adding to the enigma surrounding protease inhibitors, links between protease inhibitors and apoptosis have been published in some tissues (*364*, *365*), but not for fetal ovaries. Decreased expression of protease inhibitors in restricted fetal ovaries would suggest an increase in caspase activity, and therefore an increase in apoptosis in restricted ovaries, resulting in fewer germ cells. However, germ cell numbers are either similar between the groups at day 55, or restricted ovaries actually have more germ cells at day 75, indicating that higher levels of apoptosis in restricted germ cells is unlikely. While it seems intuitive that decreased protease inhibitor expression leads to increased apoptosis, it is interesting to note that in immature rats, at a developmental time similar to late gestation in sheep, serine protease inhibitors have been shown to impair follicle development (*366*). This illustrates the potential for a counter intuitive process whereby down-regulation of protease inhibitors may have possible beneficial consequences for ovarian development.

This dilemma may be resolved by the identification of which ovarian cells express genes for protease inhibitors. In adult bovine follicles, expression of serpins appeared to be in either granulosa and/or thecal cells (*367*). Aside from germ cells, invading cells from the regressing mesonephros also show significant apoptosis, and at least some of the protease inhibitors have been linked to mesonephric (and metanephric) development (*368*, *369*). Should invading mesonephros cells be the site of protease inhibitor gene expression, this would offer an explanation for the divergent results between decreased protease inhibitor gene expression, but no increase in germ cell apoptosis.

The fold changes observed at day 75 are less than those observed at day 55, indicating that expression levels of genes in restricted ovaries are returning to levels similar to maintenance animals. This pattern is the same for down-regulated genes at day 75. While many genes are still significantly down-regulated at day 75, the fold changes are much smaller than those observed at day 55, with the largest negative fold change at day 75 being -5.1. At least four protease inhibitors remain significantly down-regulated (*AMBP*, *AHSG*, *PLG*, and *SERPINA1*). Given the seemingly counter intuitive positive effects that down-regulation of these genes has on postnatal follicle development (*366*), their potential role in follicle formation seems worthy of further study, particularly as their expression level appears to be responsive to nutritional status.

Two members of the albumin carrier protein family also remain significantly down-regulated at day 75 (*ALB* -4.0 and *AFP* -4.2). The possible effects of these down-regulated genes previously highlighted at day 55 are likely to still be apparent at day 75. Further, given that levels of both meiosis and apoptosis of germ cells are higher at this age, and that follicles are being formed, the implications of any effects for ovarian development are likely to be more significant at this age.

This discussion has largely focused on germ cell development thus far. However, as highlighted in Section 2.4, recruitment of granulosa cells during fetal development may play an important role in the effects observed at 19 months of age. It is interesting to note that classical granulosa cells markers such as *FOXL2* and *AMH* are absent from the list of differentially expressed genes at both day 55 and day 75. Many of the differentially expressed genes are expressed in all cells, including granulosa cells and pre-granulosa cells (Tables 6.5 and 6.8). Therefore, direct effects of differentially expressed genes on pre-granulosa cells is a possibility. Differentially expressed oocyte genes also have the potential to affect the granulosa cells, with *GDF9* being a good example. *GDF9* has been extensively studied in adult ovaries, where it has been shown to be critical for follicle development and proliferation of follicular granulosa cells (*370, 371*). In the fetal ovary, *GDF9* is known to be important in follicle formation. Expression of *GDF9* has been observed prior to follicle formation in fetal ovaries from human (*336*) and sheep (*372*), but its function at these relatively early ages is still unclear. In this study, up-regulation of *GDF9* at day 55, well before follicle formation begins, is likely to be important, but its implications are unable to be determined based on the current literature.

Collectively, this data has identified temporal changes in gene expression due to under nutrition and allows the emergence of new hypotheses to understand the relationship between the nutrition regime and the results observed, thereby fulfilling the second aim of this study. In considering the data and the points raised in this discussion, two potential hypotheses are emerging.

Firstly, the alterations to the pattern of gene expression observed at day 55 appear to be mechanisms to allow the ovary, and in particular the germ cells, to survive in a low nutrition environment. With a sudden shift from low to normal nutrition, there appears a small window (or lag phase), where gene expression remains set for a low nutrition environment. Conceivably, during this window or lag phase, germ cells are able to increase in number through a mechanism as yet to be identified. The inability to detect this mechanism in this study suggests that this lag phase, where the mechanism may be active, lies between the two ages studied, day 55 and day

75. One possible mechanism appears to be increased germ cell production of nitric oxide, allowing more germ cells to survive through meiosis and apoptosis.

Secondly, direct effects of differentially expressed genes (e.g. protease inhibitors) on the function of ovarian cells up to day 75 may account for the differences observed in the day 75 ovaries, and potentially flow through to the differences observed in adult animals.

Further work is required on the cell specific expression pattern and the roles of many of the differentially expressed genes in ovarian development to elucidate the precise mechanism.

Chapter 7 . Final Discussion

This study has, for the first time, demonstrated that alterations to gestational nutrition in dams can increase indicators of fertility in female offspring. The observed changes in fetal ovarian development and fetal gene expression are consistent with the first hypothesis that restricted maternal nutrition will lead to changes in fetal ovarian gene expression and fetal ovarian morphology. However the finding of increased fertility in the female offspring requires that the second hypothesis, that restricted maternal nutrition will lead to reduced fertility in female offspring, be rejected.

However, this result potentially opens a new direction of research in the area of gestational nutrition and postnatal fertility. While the focus of current studies is aimed at understanding how restricted gestational nutrition negatively impacts postnatal fertility, the concept of improving lifetime fertility through manipulating gestational nutrition has major implications, particularly in the livestock industry. The results of this study would suggest that this concept is feasible.

Potential reasons why this study has produced results which contrast to previous studies will be discussed in Section 7.1. One of the strengths of the current study is the combination of data from whole animal physiology, endocrinology (maternal, fetal and adult offspring), morphological fetal ovarian development, and differential gene expression in the fetal ovary. How these data support each other will be discussed in Section 7.2. Unequivocal identification of the mechanism(s) underlying the relationship between gestational nutrition and postnatal fertility remain elusive. However, the data offers some insights into the mechanism(s) underlying the effects seen in the current study. These will be discussed in Section 7.3. Additionally, whether the data offers some insights into the negative effects seen in other studies will also be discussed. While the variety of data is a strength of this study, there are limitations to this study. These will be discussed in Section 7.4. From the limitations and the results, unanswered questions and future directions will be discussed in Section 7.5.

7.1 Why do the results contrast with previous studies?

Prior to the current study, work examining gestational nutrition varied markedly (summarised in Table 1.1, Section 1.3). Arguably, at a simplistic level, one could suggest that the results from the current study represent yet another variation in a field where the number of possible variables affecting the outcome is considerable. These variables include the timing of nutrition,

amount of restriction (or over feeding), type of restriction (protein, metabolisable energy), and species studied. In general, negative effects on the fertility of female offspring have been reported where over or underfeeding was imposed in mid or late-gestation. Where nutrition restriction has been applied during early to mid-gestation, differences in ovarian development have been reported, but negative effects on the fertility of the offspring are generally not reported in these studies. Thus, the timing of nutrition restriction in the current study is likely to have contributed in some part to the novel results.

Additionally, the level of nutrition restriction used in this study (60% of maintenance, based on metabolisable energy requirement) is less than that applied in most studies, where in sheep, 50% of maintenance is commonly used. As discussed in Section 2.4, the consequences of this seemingly small change in the level of nutrition restriction are substantial for the ewe. In the current study, ewes lost an average of 7% of body weight, while in a similar study using 50% of maintenance, ewes lost 16% of body weight (*177*). While the implications of the differences in maternal body weight loss for fetal ovarian development are unclear, it does nonetheless represent an additional point of difference between the current study and most other studies in sheep.

The change from 0.6 of maintenance to ad libitum feeding (combined with changing from an indoor diet to pasture), also represents a point of difference from most other studies. This factor has been highlighted throughout this thesis.

Without further studies changing the timing and level of nutrition restriction, it is not possible to definitively give a reason for the novel results observed in the current study. However, it is hypothesised that the combination of the three factors listed above (duration of restriction, amount of restriction, and change of diet) have all contributed to the results observed.

7.2 Re-examination of the data from a global perspective

Two recurrent themes are present in the data throughout this thesis.

Firstly, the differences observed appear to be ovarian driven. Differences in OR and especially AFC (Section 2.3.6) support this contention. While AFC includes follicles greater than 2 mm, and these follicles are gonadotrophin responsive (*101*), the data is consistent with a strictly ovarian response. If differences in AFC were the result of changes to the hypothalamic-pituitary system, one may have expected to see higher concentrations of FSH driving the increased follicular growth. At the time of ultrasound scanning however, the opposite was true, with lower

concentrations of FSH present in ewes from restricted dams (Section 3.4.9). The timing of scanning coincided with the appearance of the first follicle wave, with this wave known to be particularly active in the production of factors such as inhibin. One interpretation of these observations is that the differences observed in FSH may be the result of increased feedback from the ovary. Further, differences in the pattern of LH secretion were not observed. With LH being a more accurate reflection of GnRH secretion (*88*), this also suggests that changes within the ovary are primarily responsible for the observed differences. Differences in the pattern of germ cell development prior to establishment of the hypothalamic-pituitary-gonad system (Section 4.3) also suggest the differences are ovarian driven. The differences in fetal ovarian development are supported by the gene expression data which highlight a different pattern of gene expression between maintenance and restricted fetal ovaries. These differences are particularly (although not exclusively) evident in genes which are expressed preferentially or exclusively in germ cells.

The second recurrent theme throughout this thesis is the importance of the change in diet from restricted indoor feeding to ad libitum pasture. In Chapter 2, the post restriction "growth spurt" observed in restricted ewes following cessation of nutrition restriction has been highlighted as a potential contributor to the observed effects. Additionally, in discussing the absence of effects in peri-pubertal animals, the concept of waves of granulosa cell recruitment was examined (Section 2.4). The possibility was raised that granulosa cells recruited during the first wave of recruitment (coinciding with the period of nutrition restriction) may be unaffected, resulting in the lack of observable differences in OR and AFC in peri-pubertal animals. Prepubertal growing follicles contain granulosa cells predominantly from this first wave of recruitment (*40*). Growing follicles from adult animals, where differences in AFC are observed, are thought to contain granulosa cells from the second wave of granulosa cell recruitment. This wave of recruitment would occur during the immediate post restriction period, again suggesting that this period of diet change and not the actual nutrition restriction alone is the critical factor in establishing the observed affects. The importance of the post restriction period is perhaps best highlighted in Chapter 4. At the cessation of nutritional restriction, no differences were observed in the morphological pattern of germ cell development (Section 4.3). However, 20 days following the change to an ad libitum pasture diet, differences became apparent, with fetal ovaries from restricted dams containing more germ cells than those from maintenance dams. While differences in gene expression were noted in fetal ovaries at day 55 (Section 6.3.2.2), the absence of measurable morphological differences in the ovaries at day 55 led to the hypothesis

that the differences in gene expression were a mechanism to allow normal ovarian development to progress in a low nutrition environment. Following the change to an ab libitum diet, gene expression takes some time to readjust, with differences (albeit with smaller fold changes than observed at day 55) still apparent at day 75 (Section 6.3.2.3). Thus, Chapters 2, 4 and 6 each suggest that it is not the restricted nutrition alone which is responsible for the affects observed in this study, but the change from restricted nutrition to ad libitum nutrition.

7.3 Potential underlying mechanisms

The data from this study is consistent with the observed results being the consequences of changes in ovarian development. From the gene expression data, there are a small number of potential mechanisms which may underlie the changes. It is accepted that these hypotheses are highly speculative. While the data from this study may be consistent with these proposed mechanisms, there is no evidence to conclude that the mechanisms discussed are indeed responsible for the changes observed in this study.

Perhaps the most promising mechanism is the arginine to citrulline and nitric acid pathway. Nitric oxide is a key compound in many cellular processes. The number of genes identified by RNAseq and pathways identified by IPA analysis that may affect the arginine to nitric oxide and citrulline reaction, is substantial and are illustrated in Figure 7.1.

The two key differentially expressed genes regulating this reaction are *PADI6* which is germ cell specific, and *NOS1* which is expressed in either germ cells, pre-granulosa cells, or both (*320, 328*). This is consistent with a germ cell effect. How nitric oxide may facilitate the increased germ cell numbers observed is not clear. Many studies have shown that nitric oxide can cause DNA damage (*373-375*). However, one could argue the dose applied in most studies is considerable. Phoa and Epe (*355*) showed no increase in oxidative DNA damage in mouse fibroblasts overproducing nitric oxide, and also concluded that nitric oxide actually afforded DNA some protection from oxidative damage caused by H_2O_2. Further, it is not actually nitric oxide which causes DNA damage, but the products of the reaction between nitric oxide and reactive oxygen species (ROS): N_2O_3 and $ONOO^-$. Arguably, this is where the vitamin C pathways may facilitate a positive effect, regulating ROS and thus the nitric oxide – ROS reaction.

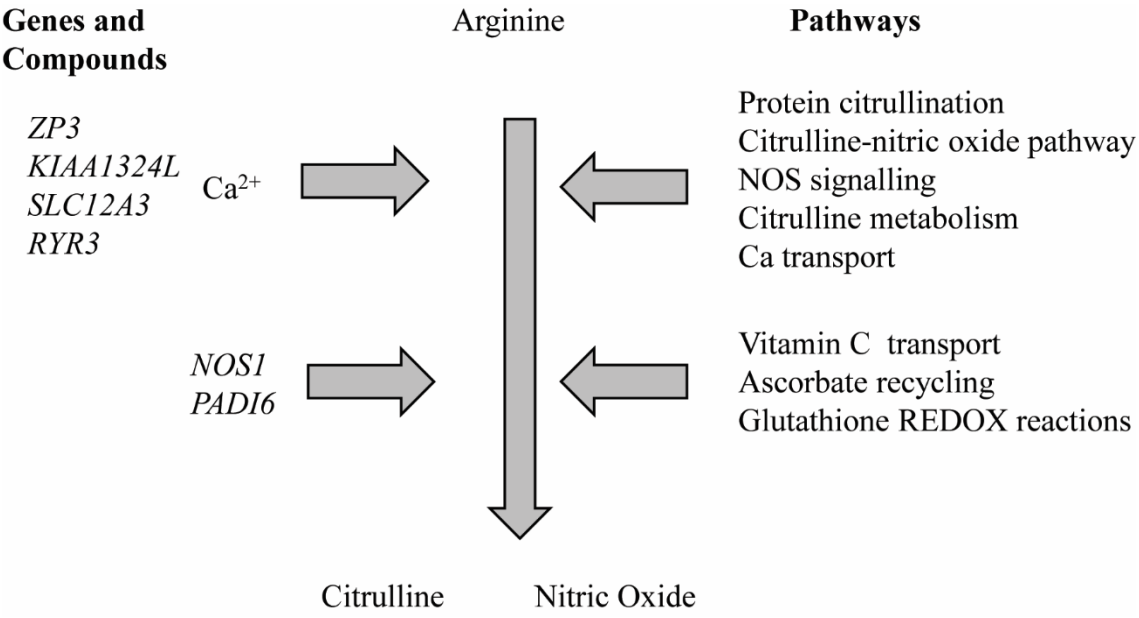

Figure 7.1 Genes and pathways involved in the arginine to citrulline and nitric oxide reaction.

While the nitric oxide pathway seems a possible mechanism to facilitate the effects of gestational nutrition on ovarian development, the presence of differential expression of a number of protease inhibitors is also of interest as a possible mechanism. The potential for protease inhibitors to have profound effects is highlighted when one considers their targets, which are proteases. Two classes of proteases with divergent roles in the fetal ovary are the caspases, (involved in apoptosis), and the proteins transcribed from the autophagy associated genes (*ATG*s) (*376*), which appear to be involved in cell survival at the gestational ages studied. There are a number of classes of protease inhibitors, those identified as differentially expressed in this study are either cysteine or serine protease inhibitors. Intriguingly, caspases are serine proteases, while ATG proteins are cysteine proteases. Thus, it is easy to hypothesise that differential regulation of protease inhibitors may have marked effects on ovarian development. However, little is known about protease inhibitors in the fetal ovary and further work is required to understand these compounds, their role, and potential as a mechanism for the effects observed in this study.

There is no evidence from the current study to suggest mechanisms involving steroids are responsible for the effects, despite their critical roles in germ cell survival and follicle formation. Steroid concentrations in fetal plasma were not affected by the nutritional regime. Further, genes involved in steroid production were not differentially expressed in fetal ovaries at either age examined.

When planning this project, the original aim was to identify possible mechanisms underlying the relationship between restricted gestational nutrition and reduced fertility in the female offspring, an outcome observed in most studies to date. The question arises then, does the current study give any insights into this relationship? One may speculate on the impacts of increases in magnitude of the fold changes observed in the current study, in response to a more severe nutrition restriction. This assumes that a higher restriction level amplifies the changes observed. Further, one could speculate on the impacts of extended differential expression of the genes in this study, in response to extended nutrition restriction. This assumes that the same genes would be affected in a longer restriction regime. When examining gene expression from comparing day 55 and day 75, 390 transcripts are switched off between day 55 and day 75 (expression value at day 75 > 0.1 fpkm, value at day 75 = 0). Additionally, using the same criteria, 476 transcripts are switched on. Any of these 476 transcripts may be differentially regulated by an extended restricted gestational nutrition and affect ovarian development. Thus, from this study, insights into mechanisms underlying the relationship between restricted gestational nutrition and low fertility in the female offspring cannot be drawn with any confidence.

7.4 Limitations of the study

The studies on adult animals found differences in OR, AFC, progesterone, and FSH. All of these measures can be classified as indicators of fertility and not fertility as such. While the progression from these indicators of fertility to actual fertility (number of lambs born) may seem logical, the potential for changes in gene expression during germ cell development and follicle formation to affect this progression is nonetheless a factor. This raises several questions: Are the oocytes in adult follicles normal? Are they capable of being fertilised? And, are they capable of producing viable embryos? Additionally, do changes during development lead to abnormal assembly of follicles in the fetal ovary? If follicles are abnormal, this may effect maturation of the oocyte during follicle growth leading to impaired fertilisation. While these scenarios cannot be discounted, three observations would suggest that fertility is likely to follow

the same trend as the indicators of fertility. Firstly, aside from differences in numbers, follicular growth appears normal by ultrasound scanning suggesting that major changes to primordial follicle assembly are unlikely. Additionally, given the recent paradigm that the oocyte drives follicle growth and differentiation (*377*), this would also suggest that at least during follicular growth, oocytes are normal. Secondly, hormone profiles are only subtly different between groups at 19 months of age, suggesting that major dysfunction in the ovary is unlikely. Finally, at 19 months of age, follicles achieve ovulation, obviously an important hurdle to overcome to achieve fertility. At laparoscopy and/or ultrasound scanning, no gross differences were observed in the appearance of corpora lutea. Thus, while all the evidence would suggest that the observed differences in indicators of fertility are likely to extend into differences in fertility, this is a projection based on the available data. As already discussed in Section 2.4, to measure fertility requires adult animals to be mated with intact rams and the number of offspring from that mating recorded. In the current study, insufficient animals were available to undertake this experiment.

The measurement of AMH in plasma was an important component of this study. Plasma concentrations of AMH have been used as an indicator of AFC, ovarian reserve and fertility (*236*). Measurement of AMH in plasma samples at the time of ultrasound scanning would have added considerably to the current study. The continued development of an AMH assay suitable for use in sheep is an ongoing project. While in some studies FSH has been used as an indicator of AFC the correlation between FSH and AFC is highly variable (*133*). A recent patent proposes a combination of FSH, inhibin, and AMH as a reliable indicator of AFC and fertility (*378*), while this seems a useful tool, without a suitable AMH assay it remains impracticable for sheep work at this stage. Concentrations of inhibin may also have provided some insights, particularly with regards to the lower FSH levels present in adults from restricted dams early in the oestrous cycle.

The differences in the pattern of gene expression are an important finding. However, a limitation of all gene expression studies is that expression differences may not necessarily be reflected in protein levels. Additionally, the role of citrulline (a product of the reaction controlled by both *NOS1* and *PADI6*) in post-translational protein modification has also been raised. Thus, within the fetal ovary, both the amount and structure of the key proteins identified as being important in this study, need further investigation.

7.5 Future directions

Given the unexpected findings from the current study, the need to replicate these results is important. Additionally, from an agricultural industry viewpoint, can these results be achieved under a restricted pasture grazing environment? In the current study, indoor feeding was selected to allow the greatest control over dietary intake for each maternal ewe. Replicating this experiment on outdoor pasture would require intensive monitoring of both pasture availability and animal condition, and would inevitably introduce some variation between animals. The weight profiles generated from the maternal animals in this study will provide a useful benchmark for monitoring animals in future studies. Additionally, the differences observed in maternal plasma progesterone concentrations will also provide another indicator of the effectiveness of any restricted pasture grazing regimes.

The timing of restricted nutrition is another issue worthy of attention. Can these effects be generated using a shorter restriction period? If so, is the important period immediately prior to day 55 of gestation or immediately after mating? Further, how much variation in the timing of dietary change (from restricted to ad libitum) is possible? In the current study, animals were synchronised and nutrition restriction ended on day 55. From a practical application standpoint, synchronisation and daily monitoring for mating marks is not feasible. Discovery of a window (ideally a 17-day window corresponding to a complete oestrous cycle) where nutrition restriction can end yet still produce the effects observed in this study, is worthy of investigation.

Extending the period of both restricted and maintenance nutrition from day 55 to day 75 (or day 90), would offer insights into both the importance of the diet change at day 55 in the current study, and the negative effects on post-natal fertility observed in other studies. The hypothesis being that genes associated with nitric oxide production will remain differentially regulated during extended restricted nutrition, but those associated with Vitamin C (or antioxidants) will not. This would potentially result in negative effects on germ cell development/numbers. The ideal time to measure these effects would be day 90 following the wave of germ cell atresia when 80% of germ cells die (*38*). Even under the current nutrition regime, examination of day 90 ovaries would be beneficial as it is unclear whether the differences in germ cell numbers represent a shift in timing of development or a more permanent change in numbers.

If one accepts the hypothesis that it is the change in diet from restricted to ad libitum which is important in establishing the observed effects, then some interesting possibilities arise. Can this effect be induced, not by nutrition restriction, but by administration of a dietary supplement at the appropriate age? The current study does give some insights into potential components of such a supplement.

The gene expression data highlights differences in the arginine, citrulline, and nitric oxide pathways in response to nutrition restriction. From this, one could speculate that arginine supplementation around day 55 of gestation may be a useful strategy. While rapid degradation of arginine in the rumen is an issue, rumen protected forms of arginine are available (*379*).

Vitamin C may also be worth considering as a supplement component. IPA identified three pathways related to vitamin C metabolism as being affected by the restricted nutrition regime at day 75. One of the properties of vitamin C is potentiation of the arginine to citrulline and nitric oxide reaction. Vitamin C is also a recognised antioxidant, providing protection against ROS (*380*). ROS, in combination with nitric oxide, produce N_2O_3 or $ONOO^-$, both of which are known to damage DNA (*373*). Thus, if the goal of a supplement was to increase nitric oxide (by arginine supplementation), addition of vitamin C may help to limit the negative effects of nitric oxide while retaining the positive effects. Ruminant animals produce their own vitamin C as, like arginine, it is rapidly degraded in the rumen. However, rumen protected forms of vitamin C are available which could be used in an oral supplement (*381*).

In Section 7.3 it was highlighted that extrapolating the data from this study to explain the relationship between restricted gestational nutrition and reduced fertility (as observed in most published studies) could not be done with any degree of confidence. The limiting factors aside, it is worth considering the following. Gestational under-nutrition appears to up-regulate the production of nitric oxide, either in, or adjacent to, the developing germ cells. Only following the change of diet, are pathways associated with vitamin C affected. If nutrition restriction were to continue for an extended period, would continued effects on the nitric oxide pathways (in the absence of effects on vitamin C pathways) result in increased germ cell DNA damage leading to reduced fertility? If this were indeed the case, would a vitamin C supplement around the time of germ cell meiosis afford some protection from negative effects of nitric oxide and help to maintain normal fertility in the offspring.

The discussion of possible mechanisms underlying the increase in indicators of fertility in female offspring highlights some potential avenues for future research. For example, the effects

of nitric oxide on germ cells requires further work. While many studies examining the effects of nitric oxide on cell function and health use exogenous nitric oxide, the approach adopted by Phoa and Epe(*355*), using fibroblast cells transfected with macrophage *iNOS,* would more closely replicate the increased concentrations hypothesized to occur in the fetal ovaries from this study. In principle, fetal germ cells could be co-cultured with *iNOS* transfected cells (either fetal ovarian cells or a mixture of fibroblasts and ovarian cells). The effects of nitric oxide on germ cells (oxidative DNA modifications) could be monitored by the alkaline elution assay described by Phoa and Epe. Establishment of a system based on that described by Phoa and Epe for use with fetal ovary cells may help to determine if there are positive effects on germ cells following exposure to a moderate increase in levels of nitric oxide. Such a system could also be used to determine whether antioxidants (e.g. vitamin C) can mitigate any detrimental effects on germ cells.

In reviewing the literature relating to the protease inhibitor genes which were identified as differentially expressed in the current study, the opportunity for further work in this area becomes apparent. Firstly, the cell specific expression of these genes (using in situ hybridisation), or protein localisation (using IHC) in the fetal ovary needs to be established. The expression of genes or localisation of proteins may give a better indication of their role in the development of the ovary, and from that, a more informed assessment of their potential contribution to the effects observed in this study. For assessment of the effects of these protease inhibitors on fetal ovary development, cell culture would seem the most viable option. This could involve culture of whole fetal ovaries (*33*) or pieces of ovaries (*270*). However, given that at day 55 and 75 fetal sheep ovaries are relatively large and a representation of all cell types would be required, then a disaggregation-re-aggregation technique as described by Dolci (*382*) may be best suited for this application.

7.6 Concluding remarks

The results from this study suggest that changes to gestational nutrition can alter developmental programming, leading to enhanced function of the reproductive system. Van de Pavert and colleagues (*383*) demonstrated in mice that maternal vitamin A levels regulate the development of the immune system (*383*). Mice whose mothers where fed a high vitamin A diet during gestation showed improved development of their immune system, and an enhanced ability to fight infection. Thus, while most studies focus on the negative implications of altered gestational nutrition, there is perhaps, potential to investigate the induction of positive effects of gestational nutrition on postnatal physiological and organ systems.

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Appendices

Appendix A. Assay protocols

A. **Procedure for the iodination of ovine Follicle Stimulating Hormone or ovine Luteinizing Hormone**
B. **Procedure for the ovine FSH Competition RIA**
C. **Procedure for the ovine Luteinizing Hormone Displacement RIA**

Reagents for iodination, RIA standard curve and RIA primary antibody are available from http://www.humc.edu/ National Hormone and Peptide program (NHPP).

1 x ovine FSH kit contains antigen, antisera and a reference hormone:

Antigen for iodination, NIDDK-oFSH-19-SIAFP (AFP4117A)

Reference hormone for the standard curve, NIDDK-oFSH-RP-2 (AFP4117A)

Antisera for the primary antibody, NIDDK-anti-oFSH-1 (AFP-C5288113)

1 x ovine LH kit contains antigen and antisera :

Antigen for iodination, NIDDK-oLH-I-4 (AFP8614B)

Antisera for the primary antibody, NIDDK Rabbit anti oLH-1 (AFP192279)

The reference hormone is an in-house highly purified ovine LH, CY1085

A. Procedure for the iodination of ovine Follicle Stimulating Hormone or ovine Luteinizing Hormone

oFSH or oLH is iodinated using the Chloramine T method and is then further purified by ion exchange chromatography.

Reagents:

- Both the ovine FSH and LH for iodination are supplied at 30 – 40 µg. This is dissolved in 0.05 M PBS (FSH pH7.2, LH pH 7.4) to a concentration of 2.5 µg/20 µL. This solution is then dispensed in 20 µL aliquots into eppendorf tubes and stored at -70°C.
- Iodine -125 radionuclide, 5mCi (185MBq) (Scimed #BD-NEZ033A005MC)
- Bovine Serum Albumin
- Chloramine T, Laboratory reagent
- Sodium metabisulphite, Laboratory reagent
- Tris (hydroxymethyl) methylamine, AnalaR
- Potassium chloride, AnalaR grade
- Hydrochloric Acid sp.gr. 1.18, AnalaR grade
- QAE-Sephadex A-25, GE Healthcare

Solutions:

- 0.05 M Phosphate Buffer (FSH pH 7.2, LH pH 7.4)
- 20 mM Tris (FSH pH 8.6, LH pH 9.0) contains Tris 0.242 g/100 mL
- 400 mM KCI in 20 mM Tris (FSH pH 8.6, LH pH 9.0) contains Tris 0.242 g/100 mL, KCI 2.982 g/100 mL. Dissolve salt in deionised distilled water and adjust pH (FSH pH 8.6, LH pH 9.0)
- 10% BSA in 20 mM Tris (FSH pH 8.6, LH pH 9.0)
- 1.6 mg/mL chloramine T in 0.05 M PBS (FSH pH 7.2, LH pH 7.4)
- 4 mg/mL sodium metabisulphite in 0.05 M PBS (FSH pH 7.2, LH pH 7.4)

Ion exchange column:

1. Swell 0.5 g QAE Sephadex A25 in 10 mL MilliQ for at least one hour.
2. Allow swollen gel to equilibrate to room temperature.
3. Add Sephadex to Poly-Prep column to give a bed volume of 1 mL. Equilibrate the column with at least 10 mL of 20 mM Tris (FSH pH 8.6, LH pH 9.0).

4. Stopper column until ready for use in the Iodination Laboratory.
5. Make up the following elution solutions as set out in the table below.

- Buffer A = 20 mM Tris (FSH pH 8.6, LH pH 9.0)
- Buffer B = 400 mM KCI in 20 mM Tris (FSH pH 8.6, LH pH 9.0)

FSH Elution solutions

Tube number	Concentration of KCL (M)	20 mM Tris pH 8.6 (mL) Buffer A	KCL 400 mM in Tris pH 8.6 (mL) Buffer B
1	0.00	3.00	0.00
2	0.10	2.25	0.75
3	0.12	2.10	0.90
4	0.14	1.95	1.05
5	0.16	1.80	1.20
6	0.18	1.65	1.35
7	0.20	1.50	1.50
8	0.22	1.35	1.65
9	0.26	1.05	1.95

LH Elution solutions

Tube number	Concentration of KCL (M)	20 mM Tris pH 9.0 (mL) Buffer A	KCL 400 mM in Tris pH 9.0 (mL) Buffer B
1	0.00	3.00	0.00
2	0.02	2.85	0.15
3	0.04	2.70	0.30
4	0.06	2.55	0.45
5	0.08	2.40	0.60
6	0.12	2.10	0.90
7	0.16	1.80	1.20
8	0.24	1.20	1.80

Chloramine T iodination and purification:

1. Thaw a 2.5 µg aliquot of oFSH or LH iodination standard. Add 0.5mCi (5 µL) of 125I-Na radionuclide.
2. Add 10 µL of 1.6 mg/mL chloramine T in 0.05 M Phosphate buffer (FSH pH 7.2, LH pH 7.4).
3. Mix and allow the mixture to react for 30 seconds.
4. Add 10 µL of 4 mg/mL sodium metabisulphite in 0.05 M Phosphate buffer, (FSH pH 7.2, LH pH 7.4) to stop the reaction.
5. Add 200 µL of 20 mM Tris (FSH pH 8.6, LH pH 9.0).

6. Transfer the mixture onto the equilibrated ion exchange column.
7. Add 2 mL of each elution buffer in order of increasing KCI concentration to the column and collect each fraction into a 12 x 75mm polystyrene tube containing 100 µL of 10% BSA in 20 mM Tris (FSH pH 8.6, LH pH 9.0).

Elution profile:

1. Transfer 5 µL of each fraction into 12 x 75 mm polystyrene tubes. Count the 5 µL fractions for 1 minute in the gamma counter.
2. Plot the counts per minute (cpm) against the concentration of KCI and keep as a record of the elution profile of the iodination. It should be consistent with previous iodinations.
3. The elution profile should have two peaks of activity. Pool the two or three fractions with highest cpm from the first peak.
4. Dispense as 800 µL fractions in Nunc Cryotubes and store at -20 ± 5°C.

B. Procedure for the ovine FSH Competition RIA

Reagents:

Primary antibody**:**

- Upon receipt, store at 1:80 dilution in assay buffer
- Before use, dilute 300 times to an initial tube dilution of 1:24,000

Tracer:

Dilute iodinated Iodine in assay buffer to give 15000 cpm/100 µL.

Second antibody pre-precipitated:

(Prepare day 1 and leave at 4°C overnight)

- Sheep anti-rabbit (in house) use at 1:35
- Normal rabbit serum (in house) use at 1:350
- Prepare 5% PEG 8000 in 0.01M PBS

Standards for FSH Competition Assay:

- Use NIDDK ovine FSH-19-SIAFP-RP-2 supplied with the FSH kit
- Supplied aliquot contains 10 µg lyophilized FSH which should be reconstituted with 1 mL distilled water

- This gives a solution containing 10,000 ng/mL = solution (A)
- Add 10.0 mL of 0.01M PBS + 0.1% BSA to solution (A) = 1000 ng/mL = solution (B)
- Distribute solution (B) into 2.0 mL aliquots and freeze = solution (C). Keep 1 aliquot for immediate dilution
- Dilute solution (C) to 20 mL and then make doubling dilutions to give 25, 12.5, 6.25, 3.12, 1.56, 0.8 and 0.39 ng/mL
- Distribute in 750 µL aliquots to give 13 standard curves. Freeze at -20°C until required

Assay Buffer:

- 0.01M PBS + 0.05M EDTA + 0.5% BSA, adjust to pH7.4.

Assay Procedure:

Day One:

Add:

- 100 µL sample /standard/control in duplicate
- 100 µL assay buffer to all tubes except total and NSB which get 300 µL (see table below)
- 100 µL tracer to all tubes
- 100 µL primary antibody to all tubes except NSB and Total
- Vortex and incubate overnight at RT

Day Two:

- Add 100 µL second antibody to all tubes except Total. Mix the antibody thoroughly before using
- Vortex and incubate on the bench for 1 hour
- Add 1.0 mL 5% W/V PEG to all tubes (except Total) and centrifuge at 3200 rpm for 35 minutes at 4°C
- Decant (except Total) and count tubes in gamma counter

	Sample volume	**Buffer volume**	**Tracer volume**	**AB1 volume**	**AB2/NRS volume**	**PEG volume**
Sample tube	100 µL	100 µL	100 µL	100 µL	100 µL	1000 µL
Std tube	100 µL	100 µL	100 µL	100 µL	100 µL	1000 µL
NSB tube	none	300 µL	100 µL	none	100 µL	1000 µL
Total tube	none	none	100 µL	none	none	none

C. Procedure for the ovine Luteinizing Hormone Displacement RIA

1. Stock Phosphate 0.5 M pH 7.4.
   - 15.6 g $NaH_2PO_42H_2O$ in 200 mL MilliQ (A)
   - 71.02 g NaH_2PO_4 anhydrous in 1000 mL MilliQ (B)
   - Add 164 mL A to B, adjust pH to 7.4
   - Store at -20°C in 40 mL aliquots

2. LH Assay Buffer.
   - 40 mL PO_4 stock
   - 37.22 g EDTA
   - 18 g NaCl
   - 2 g NaN_3
   - 2 g BSA (add last)
   - Adjust to pH 7.0 and make up to 2 litres

3. Normal Rabbit Serum (NRS).
   - Dilute 1:500 in assay buffer
   - Add to NSB 100 µL/tube

4. LH Wash buffer.
   - 40 mL PO_4 stock
   - 18 g NaCl
   - 2 g NaN_3
   - 4 mL NRS
   - Adjust to pH 7.0 and make up to 2 litres

5. Primary Antibody NIDDK Rabbit anti oLH-1.
   - Stored at 1:100
   - Dilute 1:600 in NRS/Assay Buffer (working dilution 1:60 000)

6. Tracer oLH I^{125}.
   - Count 5 µL
   - Add tracer to assay buffer in dilution of 30000 cpm/100 µL (calculated as for FSH assay)

7. Second Antibody generated in house.
   - Dilute sheep anti-rabbit 1:40 (SAR) in assay buffer

8. Standard for ovine LH Assay.

- Use CY1085 aliquots of 10 µg. Make dilutions of 20, 10, 5, 2.5, 1.25, 0.625 and 0.3125 ng/mL in assay buffer
- Distribute in 1000 µL aliquots and store at -20°C

Assay Procedure:

	TC	NSB	Std	Sample	Time
Assay Buffer	---	200 µL	100 µL	200 µL	Day 1
Filler (OVX serum)	---	100 µL	100 µL	---	Day 1
Sample/Std	---	---	100 µL	100 µL	Day 1
1°Ab (#2)	---	---	100 µL	100 µL	Day 1
NRS/Buffer	---	100 µL	---	---	Day 1
Tracer	100 µL	100 µL	100 µL	100 µL	Day 2
2°Ab (SAR10)	---	100 µL	100 µL	100 µL	Day 3

- Day 1, after adding buffer, sample, filler, NRS and antiserum (#2), vortex (lid on) and incubate overnight at 4°C
- Day 2, add the tracer, vortex and incubate overnight at 4°C
- Day 3, add second antibody, vortex and incubate overnight at 4°C
- Day 4, add 1 mL of wash buffer to all tubes except TC
- Centrifuge at 3200rpm at 4°C 35 minutes (not TC)
- Gently decant tubes and count

IBL Progesterone Coat-a-Count Kit Protocol

- Standard concentrations may vary between batches, but are in the order of 0, 0.12, 0.90, 3.00, 7.90, 15.00, 36.00 ng/mL
- Label tubes. Tubes 1-4 should be regular laboratory uncoated tubes for total counts (1 & 2) and NSB (3&4)
- Vortex all samples, calibrators and controls, use while still cold
- Each assay should have high, med and low internal controls
- Set up a 100 tube assay as per table, 20 tubes at a time

Tubes		Sample	Standard	Tracer
1-2	Total counts			500 µL
3-4	NSB		0 std, 50 µL	500 µL
5-18	Standards		50 µL	500 µL
19-24	QC samples	50 µL		500 µL
25-194	Experimental samples	50 µL		500 µL
195-200	QC samples	50 µL		500 µL

- Gently shake tube to liberate air bubbles
- Wrap in foil and incubate overnight at 4°C
- Dilute wash solution 1:70 with distilled water and mix thoroughly
- Except total counts, tip off liquid and blot dry
- Except total counts, wash tubes with 3 mL of wash solution
- Let stand for 2 minutes
- Tip off liquid and blot dry
- Count

Millipore Multi species Leptin RIA

- Set up assay as per the table
- Supplied standards are at 0, 0.78, 1.56, 3.13, 6.25, 12.5, 25 ng/mL

Tube	Sample	µL sample	µL buffer	µL antisera	Tracer	µL 2°Ab
1-2	TC				50	500
3-4	NSB	50	100	0	50	500
5-18	Standards	50	50	50	50	500
19-20	Kit QC sample	50	50	50	50	500
21-28	qc samples	100	0	50	50	500
28-xx	Experimental samples	100	0	50	50	500
Xx- end	qc samples	100	0	50	50	500

Day 1. Add sample, standard, buffer and antisera.

Mix, cover, incubate 20-24 hours at 4°C.

Day 2. Add tracer.

Mix, cover, incubate 20-24 hours at 4°C.

Day 3. Add 2°Ab (precipitation reagent).

Mix, incubate 20 minutes at 4°C.

Centrifuge at 2500 g for 20 minutes at 4°C.

Decant supernatant and count pellet remaining.

Appendix B. Fixation and histology processing protocols

Solutions

Solution A. 8% Paraformaldehyde:

Add 16 g paraformaldehyde to approximately 180 mL water.

Heat to 60 $^{\circ}$C while stirring.

While at 60 $^{\circ}$C add 5 M NaOH drop wise until solution clears. Make up to 200 mL with water.

Solution B. Double Strength Phosphate Buffered Saline (0.2 M):

For 500 mL

NaCl……...8.0 g

KCl…….0.2 g

Na_2HPO_4……1.15 g

KH_2PO_4……..0.2 g

On the day of use mix solutions A and B, 1:1 and pH to 7.4.

Fix overnight at 4°C.

Histology processing schedule

80% Alcohol	1.50 hours
95% Alcohol	1 hour
95% Alcohol	1 hour
Absolute Alcohol	1.50 hours
Absolute Alcohol	1.15 hours
Absolute Alcohol	1.15 hours
Abs Alcohol/Xylene	1.50 hours
Xylene	1 hour
Xylene	1 hour
Wax	1.50 hours
Wax (with vacuum)	2 hours

Appendix C. H&E staining protocol

Haematoxylin and Eosin
(For 5µm Paraffin Sections)

Procedure

1. Deparaffinise and hydrate sections to running tap water. Stain Nuclei in Gill's Haematoxylin. 4 minutes
2. Wash well in running tap water.
3. Blue in Scott's tap water. 1 minute
4. Wash in running tap water. 5 minutes
5. Counter stain in Alcoholic Eosin. 3 minutes
6. Wash well in running tap water.
7. Dehydrate, briskly in two changes of 95% Alcohol then two changes of 100% Alcohol, leaving in last 100% Alcohol. 2 minutes
8. Clear in three changes of Xylene.
9. Leave in last Xylene. 5 minutes
10. Mount in DPX.

Results

Nuclei	Blue
Other elements	Varying shades of pink

Solutions

Gill's Haematoxylin No. 3

Haematoxylin	6 g
Distilled Water	690 mL
Ethylene Glycol	250 mL
Sodium Iodate	0.6 g
Aluminium Sulphate	52.8 g
Acetic Acid	60 mL

Add acid after all solids have dissolved. Mix in order given. Can be used at once. Requires no differentiation. Maintain acid content by adding 1 drop of acetic acid per 100 mL of stain, do this weekly. Change solution monthly.

Alcoholic Eosin

1% aqueous Eosin (C. I. 45380)	100 mL
1% aqueous Phloxine	10 mL
95% ETOH	880 mL
Acetic acid	5 mL

Scotts Tap Water

Sodium Bicarbonate	7 g
Magnesium Sulphate	40 g
Distilled Water	2 litres

Appendix D. IHC protocols

Standard deparaffinisation, hydration, rehydration and clearing procedure

Solution	Time
Xylene	10 min
Xylene	10 min
Abs EtOH	10 secs agitating
Abs EtOH	5 min
Abs EtOH	5 min
70% EtOH	5 min
70% EtOH	5 min
TBS	5 min
TBS	5 min
IHC protocol starts here	
Deionised water	2 min
2% Methyl Green	
TBS	5 min
TBS	5 min
TBS	5 min
70% EtOH	5 min
70% EtOH	5 min
95% EtOH	5 min
95% EtOH	5 min
Abs EtOH	5 min
Abs EtOH	5 min
Xylene	10 secs agitating
Xylene	10 min
Xylene	10 min
Coverslipping	

IHC procedure for Ki-67 and MAPLC3 using Dako envision system

	MAPLC3	Ki-67
	Reagents	
1°Ab	Santa Cruz 271625 1:100 dilution	Dako M7240 1:50 dilution
Antibody diluent	Dako S3022	Dako S3022
-ve control reagent	Dako X0944	Dako X0931
Antigen retrieval solution	10 mM citrate buffer pH 6.0	10 mM EDTA, 5% Tween20 pH 8.0
	Procedure	
Reagent	Time/temp	Time/temp
Antigen retrieval	2x 5 min at 95°C	2x 10 min at 95°C
Cool	20 min	20 min
Peroxidase block	5 min	5 min
TBS	2 x 5 min	2 x 5 min
1°Ab (or –ve control reagent)	30 min	30 min
TBS	2 x 5 min	2 x 5 min
Peroxidase labelled polymer	30 min	30 min
TBS	2 x 5 min	2 x 5 min
DAB/Substrrate/Chromogen	10 min	7 min
TBS	5 min	5 min
TBS	5 min	5 min

Protocol for TUNEL

Reagent	**Time/Temp (RT unless specified)**
Proteinase K (1:50)	15 min @ 37°C
Deionised water	2 x 2 min
3% H_2O_2	5 min
PBS	1 min
TdT labelling buffer	5 min
Labelling reaction	60 min @ 37°C
TdT stop buffer	5 min
Deionised water	2 x 5min
Strep-HRP	10 min @ 37°C
PBS	2 x 2min
DAB	3.5 min
Deionised water	4 x 5 min

Appendix E. PCR protocol for sex determination

Sex Determination of Embryos by PCR analysis with *SRY*-specific primers

Reagents:

- Platinum Hot Start PCR 2X Master Mix (Invitrogen Cat 13000-013, Thermo Fisher Scientific, Auckland, NZ)
- Primers were sourced from Intergrated DNA Technologies via Thermo Fisher Scientific

- SRY specific primers (sheep)
  Use primers at a final concentration of 10 pmol/µL

  SRYfor (5' to 3') TGA AAG ACG ATG TTT ACA GTC CAG C
  SRYrev (5' to 3') AAT CAC GGA CTG GGA GCG GCT TAA T
  Product size 527 bp

- β-actin primers
  β-actin forward (5' to 3') ATG AAG ATC CTC ACG GAA CG
  β-actin reverse (5' to 3') GAA GGT GGT CTC GTG AAT GC
  Product size 420 bp

- TAE Electrophoresis Buffer 50x TAE Stock

242 g	Tris
57.1 mL	Glacial Acetic Acid
37.2 g	EDTA

  Milli-Q to 1L
  Dilute stock in Milli-Q to 1x

- DNA Loading Dye

Glycerol	500 µL
0.5M EDTA	100 µL
Bromophenol Blue 1%	125 µL
MilliQ Water	275 µL

- DNA gels
  2% Agarose containing 10 µL of 10 mg/mL ethidium bromide /100 mL of agarose

Each PCR reaction contains

Reagent	**Volume (µL)**
2X Master Mix	12.5
Forward primer	1
Reverse primer	1
Nuclease free water	9.5

PCR Programs (Eppendorf Master Cycler)

1	95 °C 10 mins	**1 cycle**
2	95 °C 20 secs	
	60 °C 20 secs	**35 cycles**
	72 °C 40secs	
3	72 °C 5 mins	**1 cycle**
4	Hold forever at 22°C	

Electrophoresis

Run electrophoresis gels at 67v for 45 minutes
Wells contain either:
5 µL sample, 3 µL loading buffer, or
3 µL marker
View on gel doc (Biorad)

Appendix F. ERCC control mixes, concentrations and expected fold changes

ERCC ID	subgroup	concentration in Mix 1 (attomoles/µL)	concentration in Mix 2 (attomoles/µL)	expected fold-change ratio	log2 (Mix 1/Mix 2)
ERCC-00002	D	15000	30000	0.5	-1
ERCC-00003	D	937.5	1875	0.5	-1
ERCC-00004	A	7500	1875	4	2
ERCC-00009	B	937.5	937.5	1	0
ERCC-00012	C	0.11444092	0.17166138	0.67	-0.58
ERCC-00013	D	0.91552734	1.83105469	0.5	-1
ERCC-00014	D	3.66210938	7.32421875	0.5	-1
ERCC-00016	C	0.22888184	0.34332275	0.67	-0.58
ERCC-00017	A	0.11444092	0.02861023	4	2
ERCC-00019	A	29.296875	7.32421875	4	2
ERCC-00022	D	234.375	468.75	0.5	-1
ERCC-00024	C	0.22888184	0.34332275	0.67	-0.58
ERCC-00025	B	58.59375	58.59375	1	0
ERCC-00028	A	3.66210938	0.91552734	4	2
ERCC-00031	B	1.83105469	1.83105469	1	0
ERCC-00033	A	1.83105469	0.45776367	4	2
ERCC-00034	B	7.32421875	7.32421875	1	0
ERCC-00035	B	117.1875	117.1875	1	0
ERCC-00039	C	3.66210938	5.49316406	0.67	-0.58
ERCC-00040	C	0.91552734	1.37329102	0.67	-0.58
ERCC-00041	D	0.22888184	0.45776367	0.5	-1
ERCC-00042	B	468.75	468.75	1	0
ERCC-00043	D	468.75	937.5	0.5	-1
ERCC-00044	C	117.1875	175.78125	0.67	-0.58
ERCC-00046	D	3750	7500	0.5	-1
ERCC-00048	D	0.01430512	0.02861023	0.5	-1
ERCC-00051	B	58.59375	58.59375	1	0
ERCC-00053	B	29.296875	29.296875	1	0
ERCC-00054	C	14.6484375	21.9726563	0.67	-0.58
ERCC-00057	C	0.01430512	0.02145767	0.67	-0.58
ERCC-00058	C	1.83105469	2.74658203	0.67	-0.58
ERCC-00059	D	14.6484375	29.296875	0.5	-1
ERCC-00060	B	234.375	234.375	1	0
ERCC-00061	D	0.05722046	0.11444092	0.5	-1
ERCC-00062	A	58.59375	14.6484375	4	2
ERCC-00067	B	3.66210938	3.66210938	1	0
ERCC-00069	D	1.83105469	3.66210938	0.5	-1

ERCC ID	subgroup	concentration in Mix 1 (attomoles/µL)	concentration in Mix 2 (attomoles/µL)	expected fold-change ratio	log2 (Mix 1/Mix 2)
ERCC-00071	C	58.59375	87.890625	0.67	-0.58
ERCC-00073	B	0.91552734	0.91552734	1	0
ERCC-00074	C	15000	22500	0.67	-0.58
ERCC-00075	B	0.01430512	0.01430512	1	0
ERCC-00076	C	234.375	351.5625	0.67	-0.58
ERCC-00077	D	3.66210938	7.32421875	0.5	-1
ERCC-00078	D	29.296875	58.59375	0.5	-1
ERCC-00079	D	58.59375	117.1875	0.5	-1
ERCC-00081	D	0.22888184	0.45776367	0.5	-1
ERCC-00083	A	0.02861023	0.00715256	4	2
ERCC-00084	C	29.296875	43.9453125	0.67	-0.58
ERCC-00085	A	7.32421875	1.83105469	4	2
ERCC-00086	D	0.11444092	0.22888184	0.5	-1
ERCC-00092	A	234.375	58.59375	4	2
ERCC-00095	A	117.1875	29.296875	4	2
ERCC-00096	B	15000	15000	1	0
ERCC-00097	A	0.45776367	0.11444092	4	2
ERCC-00098	C	0.05722046	0.08583069	0.67	-0.58
ERCC-00099	C	14.6484375	21.9726563	0.67	-0.58
ERCC-00104	B	0.22888184	0.22888184	1	0
ERCC-00108	A	937.5	234.375	4	2
ERCC-00109	B	0.91552734	0.91552734	1	0
ERCC-00111	C	468.75	703.125	0.67	-0.58
ERCC-00112	D	117.1875	234.375	0.5	-1
ERCC-00113	C	3750	5625	0.67	-0.58
ERCC-00116	A	468.75	117.1875	4	2
ERCC-00117	B	0.05722046	0.05722046	1	0
ERCC-00120	C	0.91552734	1.37329102	0.67	-0.58
ERCC-00123	A	0.22888184	0.05722046	4	2
ERCC-00126	B	14.6484375	14.6484375	1	0
ERCC-00130	A	30000	7500	4	2
ERCC-00131	A	117.1875	29.296875	4	2
ERCC-00134	A	1.83105469	0.45776367	4	2
ERCC-00136	A	1875	468.75	4	2
ERCC-00137	D	0.91552734	1.83105469	0.5	-1
ERCC-00138	B	0.11444092	0.11444092	1	0
ERCC-00142	B	0.22888184	0.22888184	1	0
ERCC-00143	C	3.66210938	5.49316406	0.67	-0.58
ERCC-00144	A	29.296875	7.32421875	4	2
ERCC-00145	C	937.5	1406.25	0.67	-0.58
ERCC-00147	A	0.91552734	0.22888184	4	2
ERCC-00148	B	14.6484375	14.6484375	1	0

ERCC ID	subgroup	concentration in Mix 1 (attomoles/µL)	concentration in Mix 2 (attomoles/µL)	expected fold-change ratio	log2 (Mix 1/Mix 2)
ERCC-00150	B	3.66210938	3.66210938	1	0
ERCC-00154	A	7.32421875	1.83105469	4	2
ERCC-00156	A	0.45776367	0.11444092	4	2
ERCC-00157	C	7.32421875	10.9863281	0.67	-0.58
ERCC-00158	B	0.45776367	0.45776367	1	0
ERCC-00160	D	7.32421875	14.6484375	0.5	-1
ERCC-00162	C	58.59375	87.890625	0.67	-0.58
ERCC-00163	D	14.6484375	29.296875	0.5	-1
ERCC-00164	C	0.45776367	0.68664551	0.67	-0.58
ERCC-00165	D	58.59375	117.1875	0.5	-1
ERCC-00168	D	0.45776367	0.91552734	0.5	-1
ERCC-00170	A	14.6484375	3.66210938	4	2
ERCC-00171	B	3750	3750	1	0

Appendix G. Nanostring primers

Gene	Accession number	Target Sequence
ACVR2B	XM_012099394.2	AGGACAAGCAGTCGTGGCAGAGTGAGCGGGAGATCTTCAGCACGCCTGGCATGAAGCACGAGAACCTGCTGCAGTTCATTGCCGCTGAGAAGCGAGGCTC
AFP	XM_004009903.2	AGCCCTGGCAAAACGAAGCTGCGGTCTCTTTCAGAAATTAGGAGAATATTACTTACAAAATGCGTTTCTTGTTGCATACACAAAGAAGGCTCCTCAGCTG
AGT	XM_012105586.2	CCTCTCTTCCTTCGCCCCAATCACTCTCCCACGCTCACTAGACTTGTCCACGGACCCAAATCTCGCTGCTGAGAAGATCAACAGGTTCATGCATTCAGCG
ahr	XM_012176453.2	CATCTTTAGAACCAAACACAAACTCGACTTCACACCTACTGGTTGTGATGCCAAAGGAAAACTTGTTTTAGGCTACACTGAAGCGGAGCTGTGCTTGAGA
AHSG	NM_001009802.1	GTGGAAGGAGACTGCGATATCCACGTGCTGAAACAAGATGGCCAGTTTTCCGTGCTGTTTACAAAATGTGATTCCAGTCCAGATTCCGCCGAGGACGTGC
ALB	NM_001009376.1	GGCTTCATCTGCCAGACAGAGACTCAGGTGTGCCAGTATTCAAAAATTCGGAGAAAGAGCTTTAAAAGCATGGTCAGTAGCTCGCCTGAGCCAGAAATTT
ALDH1A1	NM_001009778.1	TGCTGATGCCGACTTGGACAATGCTGTTGAATTTGCACACCAAGGAGTATTCTATCACCAGGGCCAATGTTGTATAGCTGCATCCCGTCTCTTTGTAGAA
AMBP	XM_004003983.3	TTCTGTTTCACAAAGCCAAATGGAATATCACCATGGAGTCCTATGTGGTCCACACCAACTACGACGAGTATGCCATTTTTCTGACCAAGAAATTGAGCCG
APOA2	XM_004002693.3	TTGAGAAGACGCAGGAGGAACTGACGCCCCTGTTCAAGAAGGCTGGGACTGACCTGCTTAACTTCTTGAGCAGTTTTATAGACCCCAAAAAGCAGCCTGC
APOC3	XM_004016048.2	ATCCCTGCTGGACAAGATGCAGGGCTATGTGAAGGAGGCCACCAAGACCGCCAAGGATGCCCTGAGCAGTGTTCAGGAGTCCCAGGTGGCCCAGCAGGCC
APOH	XM_004013155.3	GCCCAGAGTATGTCCTTTTGCTGGGATCTTAGAAAACGGAACGGTACGCTATACAACGTTTGAGTATCCCAACACGATCAGCTTTTCTTGCCACACTGGG
BMPR2	NM_001306123.1	AGAACATTTCCTCTGAGCATTCGATGTCCAGCACACCTTTGACTATAGGGGAAAAGAACCGAAACTCAATTAACTATGAACGGCAGCAAGCACAAGCTCG
CCNB1	XM_004016916.3	TATCTATGCTTATCTAAGACAACTTGAGGAAGAGCAAGCAGTCAAACCAAAATACCTAATGGGTCGTGAAGTCACTGGAAACATGAGAGCCATCCTGATT
CRABP2	XM_012183447.1	GGTCGGAGAGGAGTTTGAAGAGCAGACTGTGGACGGGAGGCCCTGTAAGAGCCTGGTGAAATGGGAGAGTGAGAACAAAATGGTCTGCGAGCAGAGGCTG
CYP11A1	NM_001093789.1	CACCGAGGCCCAGAAGTTCATTGATGCCGTCTACAAGATGTTCCATACCAGTGTCCCTCTGCTCAACCTCCCTCCAGAACTGTACCGTCTGTTCAGAACC

Gene	Accession number	Target Sequence
CYP17A1	NM_001009483.1	CAGCAAAGCCATGGAAAAGATGAAGGGTTGTGTTGAAACGCGAAATGAATTGCTGAGTGAAATCCTTGAAAAA TGTCAGGAGAACTTCACCAGCGACTCC
DACH1	XM_012184753.2	GGACAACCACTACCTCCAGGATTCCCATCTCCTTTTCTGTTTCCTGATGGATTGTCTTCCATAGAGACCCTTCTGA CTAACATACAGGGGCTCTTGAAAG
DAZL	XM_015092718.1	CCAGAGAATAGACTGAGAAACTCCGTTGTCACTCAAGACGACTACTTCAAGGATAAAAGAGTTCATCACTTTAG AAGAAGTCGGGCAGTGCTTAAGTCTG
FETUB	XM_004003061.3	CCCTGACTCTGTGCCTGTTGGTATTTGCCATGGTTCTCTGAGTCAATCACAAGGAAACCCGGGCAAGACGATCTC CGTAACTTGCTCCTTCTTTAATTCA
FIGLA	XM_015094077.1	ATAGCAGCAATACTTCTGAGCCACATACATCCTCAGCTAGAGAGCTATCGAGAATTATACAACGTGCCGGCTGT GCTATGGGCTTGAAGAATGAGAAGGA
FSHR	NM_001009289.1	CTTCGCAAACTGGAGGCGGCAAACCTCTGACCTTCATCCAATTTGCAACAAATCTATTTTAAGGCAAGAAGTTGA TGACATGACTCAGGCTAGGGGTCAG
FSTL4	XM_012178481.2	TTGCAAGTTTCCTAGTATAAGGTATGCGCTGCTAACGAGATCGGGTGGGTTTTAGGATGTAGGATTTCTGCCTTG GGCTGCGATGAGAACCTGCTGCCAT
g6pd	NM_001093780.1	GCAGTGATGAACTGCGGGAGGCCTGGCGGATCTTCACTCCACTGCTCCACCACATCGAACGCGAGAAGGCCCGG CCCATCCCCTACGTGTATGGCAGCCG
gapdh	NM_001190390.1	GCGCCAAGAGGGTCATCATCTCTGCACCTTCTGCTGACGCTCCCATGTTTGTGATGGGCGTGAACCACGAGAAGT ATAACAATACCCTCAAGATTGTCAG
GC	XM_004009899.3	ATGTCCAGTTGCCCACGAACAAAGATGTGTGTGATAAAGGAAACACCAACGTCCTGGATCAGTATATATTTGAG CTAAGCAGGAAGACTCAGATTCCAGA
GDF9	NM_001142888.2	GGGACAGCCTGTTTAACATGACTCTTCTCGTAGCGCCCTCACTGCTTTTGTATCTGAACGACACAAGTGCTCAGG CTTTTCACAGGTGGCATTCCCTCCA
GRIA1	XM_012178832.2	GGGATAACTCAAGTGTCCAGAATAGAACATACATCGTCACCACAATCCTAGAAGATCCTTATGTGATGCTCAAG AAGAATGCCAACCAGTTTGAAGGCAA
HSD3B	NM_001135932.1	AAAAGGTCCCGAACATACAAGGACAGTTCTACTACATCTCAGACGACACACCACACCAAAGCTATGATGACCTC AATTACACTTTGAGCAAAGAATGGGG
ILO-15L	XM_012190396.2	ATAATCTATTGGTGTGTCCTGCTGAGACTCTCCGTTGCTTCCGGCTGGAGCTGTCTGTGATTGGGTTTGAGGAGG GCCCATCCGTGGGGATTGTTGTGTT
INHA	NM_001308579.1	TCCGGCCATCCCAGCACACACGCAGCCGCCAGGTGACTTCGGCCCAGCTGTGGTTCCATACTGGACTGGACAGA CAGGAGACCACTGCCACCAATAGCTC
INHBA	NM_001009458.1	CGGGGCTTGGAGTGTGACGGCAAGGTCAACATTTGCTGTAAGAAACAGTTCTATGTTAGTTTCAAGGACATTGG CTGGAATGACTGGATCATCGCTCCCT
INSL3	NM_001024738.1	GCGGCTGGCGGCGACCGTGAGCTCCTACGGTGGCTGGAAGGACAACATCTCCTCCATGGGCTGATGGCCAGTGG GGACCCCGTGCTGGTACTGGCCCCAC
ITIH2	XM_004014178.3	AGTCTTGGAAACCCTGGCCGAGATGGATGGTTTGGAGGATTTTCTATCCAAGGACAAGCATGCAGACCCTGATTT CACCAAGAAATTGTGGGCCTATTTG

Gene	Accession number	Target Sequence
ldlr	XM_004008531.3	AAGCCATTTTTAGTGCCAACCGCCTCACAGGCTCAGACATCAGTCTGATGGCAGAAAACCTGTTATCGCCGGAG GACATTGTCCTTTTCCACAACCTCAC
LHR	NM_001278566.1	TTCCGCCATCTTTGCTGAGAGTGAACTGAGTGGCTGGGATTATGACTATGGTTTCTGCTTACCCAAGACACTCCA GTGTGCTCCTGAACCAGATGCTTTC
MGST1	XM_004006818.3	CGTGTTCGAAGAGCCCACTTGAATGACCTTGAAAACATCGTGCCATTTCTTGGTATTGGCCTTCTGTATTCCTTGA GTGGTCCAGACCTCTCTACAGCCA
nobox	XM_012177321.2	GCCAGTGGCCTTCATAAGCAGTAAACTGTCTCCTCTTGGGTTTCTCTCTCTGTAGTCACATGTCTCCCTTCTCACG CTAGTGAAGACCACCATGCCCTTC
NOS1	XM_004017589.3	ACGTCCGCACAAAAGAACAGCTCTTTCCTCTTGCCAAAGAGTTCATTGATCAGTACTACTCATCAATTAAAAGGT TCGGCTCCAAAGCCCACATGGAGAG
PADI6	XM_012153966.2	AGAGCTTGGAAAATGGCTTTCAGCAGAGAAGTTACTGAGGGCCCATTAAAATCCTGGCTGTTCTGACTGCACCTT GGCCGTGATCTTGTTTGGGGCGGGG
PLAC1	XM_012107306.2	TGTTGAACAACGAAGTATATGTACACTTCCATGAGTTACACTTGGGCCTGGGTTGCCCTGCCAACCATGTTCAGC CATATGCCTACCAATTCACCTATCG
RARA	XM_012186063.1	GGACCTGGAGCAGCCAGACAGGGTGGACATGCTGCAGGAGCCGCTGCTTGAGGCACTGAAGGTCTATGTGCGG AAACGGAGGCCCAGCCGCCCACACATG
RARRES2	XM_015095437.1	CGGAAGAAGGACTGGAGGAAAGCAGACTGCAAGGTCAAGCCCAATGGGAGAAAGCGGAAATGCCTGGCCTGCA TCAAGCTGGACTCAAAGGATCAAGTCC
sdha	XM_012097183.1	GCTGGGGAAGAATCTGTCATGAATCTTGACAAATTGAGATTTGCCGATGGAAGCATAAGAACATCGGAATTGCG ACTCAGCATGCAGAAGTCGATGCAGA
SERPINA1	NM_001009799.1	ATCAACTTCAGGGATGCTGAGGAGGCCAAGAAGAAGATCAATGATTATGTAGAGAAGGGAAGCCATGGAAAAA TTGTGGATTTGGTAAAGGATCTTGACC
SPP2	NM_001009453.1	TTGGGGATATCTTGGGATCCTCTACATCAAGAAACAGTCACCTGCTTGGCCTCACTCCTGACAGATCCAGAGGTG AACCGCTTTATGAACGATCACGTGA
STAR	NM_001009243.1	GGGACAGGGTGGTAGCGCATTTTCAGTAAGATACTACAGCTCAGCTACTACAGTAGCATTTTAGTACCAAGAGA ATCGGGGACAAGGCTCTTCGAACTTC
ywhaz	NM_001267887.1	GAAAAGTTCTTGATCCCAAACGCTTCACAAGCAGAGAGCAAAGTCTTCTATTTGAAAATGAAAGGAGACTACTA TCGCTACTTGGCTGAGGTTGCAGCTG
zp2	XM_015104114.1	TTATCCTGAGTGTGTCTGCGAGTCCACAGTTTCTATAGTTTCAGGGAAGCTGTGTACTCAAGATGGGTTTATGGA CGTCAAGGTCTACCGCCATCAAACA
ZP3	XM_004020981.1	AGCCACGTGCCCCTGCGACTGTTCGTGGACCACTGCGTGGCCACACTGACGCCAGACTGGAGCACCTCGCCTTAC CACACCATCGTGGACTTCCACGGCT
zp4	XM_004021373.3	GACAGTGAATGTAAACCTGTGATGGCAACACACACTTTTGTTCTGTTCCGGTTTCCATTTACTACCTGTGGTACTA CAAAACAGATGACTGGGAAGCAGG

Appendix H. Day 55 differentially expressed genes

Gene ID	Gene	Restricted fpkm	Maintenance fpkm	log2(fold change)	Test stat	p value	q value
ENSOARG00000009954	-	2.35431	0.301884	-2.96324	-3.62567	5.00E-05	0.013907
ENSOARG00000010604	-	0	2.03906	inf		5.00E-05	0.013907
ENSOARG00000017575	-	1.49058	5.46257	1.8737	3.68223	5.00E-05	0.013907
ENSOARG00000019994	-	21.6299	6.12529	-1.82017	-4.45544	5.00E-05	0.013907
ENSOARG00000008517	-	6.13368	12.8323	1.06496	1.93129	5.00E-05	0.013907
ENSOARG00000008061	-	33.7653	19.7285	-0.77526	-1.61402	5.00E-05	0.013907
ENSOARG00000017859	-	42.6436	25.1198	-0.76351	-3.83172	5.00E-05	0.013907
ENSOARG00000000617	-	11.458	28.9567	1.33754	1.99478	5.00E-05	0.013907
ENSOARG00000004939	-	94.9776	54.9841	-0.78857	-2.44909	0.0001	0.025456
ENSOARG00000020259	-	187.425	86.2568	-1.1196	-1.95851	5.00E-05	0.013907
ENSOARG00000011986	-	164.821	98.0049	-0.74997	-1.72592	0.00015	0.034134
ENSOARG00000009299	-	372.221	713.806	0.939375	2.10516	5.00E-05	0.013907
ENSOARG00000007855	-	2432.45	1279.02	-0.92737	-2.19929	5.00E-05	0.013907
ENSOARG00000012079	-	1193.4	1909.5	0.678119	1.66424	0.00015	0.034134
ENSOARG00000016430	ACACB	5.08823	2.67092	-0.92983	-1.71305	5.00E-05	0.013907
ENSOARG00000001408	ACVR2B	3.52827	6.83484	0.953948	2.58695	5.00E-05	0.013907
ENSOARG00000013966	AFP	0.622928	30.0446	5.5919	7.64149	5.00E-05	0.013907
ENSOARG00000003055	AGT	0.691858	3.91933	2.50206	2.13353	0.0002	0.043533
ENSOARG00000020528	AHSG	10.4652	184.878	4.14291	5.44999	5.00E-05	0.013907
ENSOARG00000013782	ALB	1.34437	16.148	3.58635	8.72235	5.00E-05	0.013907
ENSOARG00000006134	AMBP	0.286185	7.20928	4.65483	2.59983	5.00E-05	0.013907
ENSOARG00000015661	APOH	1.54216	10.401	2.75371	2.51958	5.00E-05	0.013907
ENSOARG00000010703	ATP2C2	8.96587	17.6561	0.977653	1.99168	5.00E-05	0.013907
ENSOARG00000000788	BAIAP2L1	33.9505	16.1308	-1.07361	-2.7145	5.00E-05	0.013907
ENSOARG00000011174	C1orf112	18.1453	29.9208	0.721555	2.13097	5.00E-05	0.013907

Gene id	Gene	Restricted fpkm	Maintenance fpkm	log2(fold change)	Test stat	p value	q value
ENSOARG00000012206	CA5A	0.587683	5.69766	3.27726	2.47812	5.00E-05	0.013907
ENSOARG00000000817	CALY	7.8467	18.0158	1.19911	2.34406	5.00E-05	0.013907
ENSOARG00000004318	CDC2	4.61195	7.756	0.749936	3.06653	5.00E-05	0.013907
ENSOARG00000009067	CHL1	3.3508	1.45061	-1.20785	-2.37123	5.00E-05	0.013907
ENSOARG00000011479	CRTAC1	19.6994	7.24429	-1.44323	-2.21369	5.00E-05	0.013907
ENSOARG00000012632	DHX40	47.7179	72.9756	0.612882	1.94524	5.00E-05	0.013907
ENSOARG00000005161	DNAH1	1.85491	0.960314	-0.94977	-1.53714	5.00E-05	0.013907
ENSOARG00000015028	DNAH9	0.987453	1.93032	0.967053	1.82872	5.00E-05	0.013907
ENSOARG00000015375	ERC2	3.0182	1.2941	-1.22174	-2.17977	0.0002	0.043533
ENSOARG00000018378	FAM3C	55.7943	33.7199	-0.72652	-3.03996	0.0001	0.025456
ENSOARG00000020526	FETUB	0.245131	10.6503	5.44119	3.60959	5.00E-05	0.013907
ENSOARG00000000291	FGB	1.1661	3.0032	1.36482	4.78969	5.00E-05	0.013907
ENSOARG00000011205	FIGLA	8.19908	0.835125	-3.2954	-2.74694	5.00E-05	0.013907
ENSOARG00000011919	FOXR1	5.50283	0.301097	-4.19187	-3.24832	5.00E-05	0.013907
ENSOARG00000002203	FUS	79.9934	51.279	-0.64151	-1.80146	0.0001	0.025456
ENSOARG00000012835	GC	0.220897	5.29353	4.58278	2.65218	5.00E-05	0.013907
ENSOARG00000013229	GDF9	9.44508	2.57939	-1.87254	-2.85607	5.00E-05	0.013907
ENSOARG00000020669	HIST2H2BF	29.8712	5.30623	-2.49299	-2.1468	5.00E-05	0.013907
ENSOARG00000018312	HPX	2.08873	6.19099	1.56754	1.89409	0.00015	0.034134
ENSOARG00000008800	HSP70	30.2673	10.7441	-1.49421	-2.53271	5.00E-05	0.013907
ENSOARG00000008817	HSP70	6.85096	30.1346	2.13704	3.19285	5.00E-05	0.013907
ENSOARG00000013662	ITIH2	0.210432	2.15385	3.35549	2.84795	5.00E-05	0.013907
ENSOARG00000014780	KIAA1324L	2.75739	0.462474	-2.57586	-3.20494	5.00E-05	0.013907
ENSOARG00000004281	NEFH	16.8981	8.95828	-0.91556	-1.84514	5.00E-05	0.013907
ENSOARG00000004210	NOS1	1.67547	0.499325	-1.74651	-1.7184	0.00015	0.034134
ENSOARG00000010997	PADI6	15.91	2.48478	-2.67875	-3.14989	5.00E-05	0.013907
ENSOARG00000004585	PNLDC1	7.81273	2.53961	-1.62122	-2.54699	5.00E-05	0.013907
ENSOARG00000008453	RIBC1	13.3927	5.82082	-1.20215	-1.76308	5.00E-05	0.013907
ENSOARG00000020791	RPS27L	247.854	114.927	-1.10878	-2.19818	5.00E-05	0.013907
ENSOARG00000002474	SAMD15	6.91406	2.41784	-1.51581	-3.76059	5.00E-05	0.013907
ENSOARG00000014882	SERPINA1	3.32337	84.8135	4.67358	5.08455	5.00E-05	0.013907

Gene id	Gene	Restricted fpkm	Maintenance fpkm	log2(fold change)	Test stat	p value	q value
ENSOARG00000015144	SERPINA5	15.2224	25.1028	0.721659	1.55178	0.0002	0.043533
ENSOARG00000003976	SH2D4A	2.51641	6.62471	1.39649	2.08323	0.00015	0.034134
ENSOARG00000006804	SPCS3	119.602	250.43	1.06616	2.56796	5.00E-05	0.013907
ENSOARG00000018567	SPESP1	5.2307	1.98085	-1.40089	-3.4209	0.0001	0.025456
ENSOARG00000019162	SPP2	0.619347	6.43414	3.37693	2.42679	5.00E-05	0.013907
ENSOARG00000001996	TADA2A	125.163	198.291	0.663805	1.81793	0.00015	0.034134
ENSOARG00000019474	TDRD15	7.16202	3.26655	-1.1326	-4.32998	5.00E-05	0.013907
ENSOARG00000016633	TMED2	152.878	417.808	1.45046	4.0733	5.00E-05	0.013907
ENSOARG00000001811	TNNI3	25.6115	12.0031	-1.09338	-1.53237	0.00015	0.034134
ENSOARG00000018004	VTN	0.481068	5.5989	3.54083	2.5518	5.00E-05	0.013907
ENSOARG00000005507	WDR87	5.40362	2.34813	-1.20241	-2.85052	5.00E-05	0.013907
ENSOARG00000003901	ZNF19	3.30003	1.83378	-0.84766	-1.96789	0.0001	0.025456
ENSOARG00000013902	ZP3	7.2791	0.124683	-5.86743	-4.14164	5.00E-05	0.013907

Appendix I. Day 75 differentially expressed genes

Gene ID	gene	Restricted fpkm	Maintenance fpkm	log2(fold change)	Test stat	p value	q value
ENSOARG00000001279	-	9.44453	27.0592	1.51857	1.84808	0.0001	0.016123
ENSOARG00000002402	-	8.83006	5.04559	-0.8074	-2.29628	0.0003	0.034708
ENSOARG00000004556	-	781.176	1169.4	0.582046	1.73145	5.00E-05	0.00913
ENSOARG00000005542	-	378.406	582.199	0.621577	1.80296	5.00E-05	0.00913
ENSOARG00000005549	-	563.981	838.408	0.572006	1.96443	0.0002	0.027064
ENSOARG00000006661	-	11.178	6.50995	-0.77995	-2.15932	5.00E-05	0.00913
ENSOARG00000008061	-	26.6971	41.2605	0.628077	1.56828	0.00035	0.039587
ENSOARG00000009868	-	14.6866	6.09321	-1.26922	-1.79615	0.00035	0.039587
ENSOARG00000009954	-	17.4997	7.92298	-1.14322	-3.29522	5.00E-05	0.00913
ENSOARG00000010944	-	639.952	994.647	0.636222	1.75352	5.00E-05	0.00913
ENSOARG00000011629	-	1.76303	10.1326	2.52287	1.8382	0.0002	0.027064
ENSOARG00000011986	-	161.868	266.109	0.717201	1.96258	5.00E-05	0.00913
ENSOARG00000012947	-	90.1067	43.8499	-1.03906	-1.96213	5.00E-05	0.00913
ENSOARG00000013149	-	81.2957	143.602	0.820825	1.56286	0.0002	0.027064
ENSOARG00000016402	-	78.3751	118.919	0.601513	1.78981	0.0001	0.016123
ENSOARG00000017111	-	517.912	908.721	0.811131	1.75298	5.00E-05	0.00913
ENSOARG00000017575	-	2.12044	4.23029	0.996392	1.9807	5.00E-05	0.00913
ENSOARG00000017944	-	34.455	51.5948	0.582512	2.23261	0.0002	0.027064
ENSOARG00000018884	-	95.389	144.912	0.603279	1.73382	5.00E-05	0.00913
ENSOARG00000019174	-	325.645	501.883	0.624048	1.58656	0.00025	0.031314
ENSOARG00000019479	-	25.4592	5.54325	-2.19939	-2.56134	5.00E-05	0.00913
ENSOARG00000019994	-	28.7556	11.7446	-1.29185	-3.28852	5.00E-05	0.00913
ENSOARG00000020231	-	52.7698	161.393	1.61279	4.86633	5.00E-05	0.00913
ENSOARG00000016430	ACACB	15.5031	10.2957	-0.59051	-1.72352	0.0002	0.027064
ENSOARG00000006156	ADAMTS14	13.3968	5.31634	-1.33338	-2.87617	5.00E-05	0.00913
ENSOARG00000013966	AFP	2.93872	12.4881	2.0873	5.94092	5.00E-05	0.00913
ENSOARG00000020528	AHSG	18.3058	108.964	2.57348	3.86726	5.00E-05	0.00913

Gene id	Gene	Restricted fpkm	Maintenance fpkm	log2(fold change)	Test stat	p value	q value
ENSOARG00000013782	ALB	4.07948	16.3494	2.00278	5.84517	5.00E-05	0.00913
ENSOARG00000012705	ALDH1A1	9.92088	18.1286	0.869725	2.20799	5.00E-05	0.00913
ENSOARG00000006134	AMBP	1.39912	9.03182	2.6905	2.50429	5.00E-05	0.00913
ENSOARG00000013137	APAF-1	24.0295	15.5152	-0.63112	-2.02888	5.00E-05	0.00913
ENSOARG00000017104	APCDD1L	7.03592	21.2088	1.59185	2.64517	5.00E-05	0.00913
ENSOARG00000010709	ARMC2	13.1026	6.92312	-0.92035	-2.17126	5.00E-05	0.00913
ENSOARG00000011996	ASB11	15.9816	8.18096	-0.96607	-2.45282	0.0001	0.016123
ENSOARG00000000788	BAIAP2L1	96.641	61.9162	-0.64232	-1.98854	5.00E-05	0.00913
ENSOARG00000014065	C11orf16	26.8497	16.6668	-0.68794	-1.60554	0.00045	0.047036
ENSOARG00000001641	C20orf27	64.5303	99.0619	0.618354	1.641	0.00035	0.039587
ENSOARG00000008549	C7orf25	39.6799	26.044	-0.60746	-1.69577	0.00025	0.031314
ENSOARG00000000193	CADPS2	23.4923	15.5127	-0.59874	-1.78384	0.00015	0.02186
ENSOARG00000003936	CALB2	8.99995	18.3736	1.02964	1.64976	0.0001	0.016123
ENSOARG00000010904	CCDC64	64.6144	43.8887	-0.55801	-1.60712	0.0003	0.034708
ENSOARG00000011731	CCDC88C	13.9998	9.04836	-0.62968	-1.77025	0.00015	0.02186
ENSOARG00000004893	CCL16	0	2.30365	inf	#NAME?	0.0001	0.016123
ENSOARG00000005718	CEP164	17.074	11.5727	-0.56107	-1.66459	0.0001	0.016123
ENSOARG00000009067	CHL1	21.8493	9.72801	-1.16737	-3.36599	5.00E-05	0.00913
ENSOARG00000017833	CHRDL1	17.7895	29.899	0.749068	1.67308	0.00025	0.031314
ENSOARG00000019611	CLIP4	13.7178	8.33321	-0.71911	-1.66939	0.00025	0.031314
ENSOARG00000019995	CNR1	7.97799	15.4845	0.956728	1.90415	0.0003	0.034708
ENSOARG00000007080	CNTNAP4	7.79422	13.7689	0.820936	2.06854	5.00E-05	0.00913
ENSOARG00000005504	COCH	36.8878	22.9595	-0.68405	-1.88365	0.00025	0.031314
ENSOARG00000002812	COX5A	472.734	301.379	-0.64945	-1.70878	0.0001	0.016123
ENSOARG00000015209	DACH1	31.4012	46.7392	0.573814	1.61597	0.00025	0.031314
ENSOARG00000018383	DHX58	18.7391	11.7385	-0.6748	-1.82158	5.00E-05	0.00913
ENSOARG00000000853	DLEC1	7.91677	4.43796	-0.83501	-1.96488	5.00E-05	0.00913
ENSOARG00000005161	DNAH1	5.54745	3.1531	-0.81505	-2.1632	5.00E-05	0.00913
ENSOARG00000009918	DOCK5	15.2172	8.13754	-0.90304	-2.59871	5.00E-05	0.00913
ENSOARG00000010103	ENTPD4	29.1735	18.6488	-0.64558	-2.27722	5.00E-05	0.00913
ENSOARG00000007009	EPB41L4B	37.6249	24.1522	-0.63954	-1.62713	0.00045	0.047036

Gene id	Gene	Restricted fpkm	Maintenance fpkm	log2(fold change)	Test stat	p value	q value
ENSOARG00000000113	FAM204A	41.5036	24.5765	-0.75596	-2.36766	5.00E-05	0.00913
ENSOARG00000006246	FAP	10.6741	18.3454	0.781301	2.28731	5.00E-05	0.00913
ENSOARG00000011205	FIGLA	45.0748	22.9697	-0.97259	-2.15599	0.0001	0.016123
ENSOARG00000011919	FOXR1	32.0543	15.3523	-1.06206	-2.24573	5.00E-05	0.00913
ENSOARG00000013615	FSTL4	12.4105	6.66113	-0.89772	-1.86402	5.00E-05	0.00913
ENSOARG00000009427	GLRX	263.993	132.171	-0.99809	-2.01433	5.00E-05	0.00913
ENSOARG00000006913	GPANK1	143.943	90.0858	-0.67612	-2.05449	5.00E-05	0.00913
ENSOARG00000012389	GPRC5B	46.8659	70.8761	0.596762	1.68601	0.00015	0.02186
ENSOARG00000016103	GREB1	28.3305	42.105	0.571634	1.75102	5.00E-05	0.00913
ENSOARG00000008232	GUCY1A3	16.0194	10.2048	-0.65058	-1.58042	0.0003	0.034708
ENSOARG00000002271	HAX1	150.078	104.337	-0.52447	-1.52832	0.0004	0.04393
ENSOARG00000007725	HIP1R	107.056	52.6993	-1.02251	-3.28444	5.00E-05	0.00913
ENSOARG00000015415	HPCAL1	111.438	168.195	0.593897	1.60274	0.0003	0.034708
ENSOARG00000007452	HSPA6	18.3515	32.9817	0.845767	1.97669	5.00E-05	0.00913
ENSOARG00000008248	HSPG2	36.8144	59.5639	0.694168	2.07812	5.00E-05	0.00913
ENSOARG00000011513	IRF8	50.8752	28.7638	-0.82271	-2.01341	5.00E-05	0.00913
ENSOARG00000007233	ISG17	89.5322	45.5016	-0.97649	-1.86511	0.0001	0.016123
ENSOARG00000008230	ITM2B	299.92	427.073	0.509907	1.70218	0.00045	0.047036
ENSOARG00000010523	JAG1	88.3776	52.5666	-0.74953	-2.19709	5.00E-05	0.00913
ENSOARG00000014631	JAK3	33.9899	49.2082	0.533795	1.59374	0.0002	0.027064
ENSOARG00000009874	KCTD9	37.4257	22.9342	-0.70653	-1.78394	5.00E-05	0.00913
ENSOARG00000013162	LGMN	169.799	257.295	0.599597	1.71884	0.00015	0.02186
ENSOARG00000012587	LHX8	17.1096	8.96052	-0.93315	-3.56015	5.00E-05	0.00913
ENSOARG00000011565	LIG1	99.4201	69.2417	-0.5219	-1.6401	0.0002	0.027064
ENSOARG00000011888	MAEL	38.188	24.9926	-0.61162	-2.32919	5.00E-05	0.00913
ENSOARG00000014746	MAP3K15	14.0655	9.27188	-0.60123	-1.54024	0.0003	0.034708
ENSOARG00000000249	MAP7	31.9318	21.3404	-0.58141	-1.63208	0.0003	0.034708
ENSOARG00000003214	MATER	7.05085	3.08533	-1.19237	-2.04573	0.00015	0.02186
ENSOARG00000014271	MOXD1	6.74105	15.7278	1.22227	2.90159	5.00E-05	0.00913
ENSOARG00000005356	MVP	134.713	92.1745	-0.54745	-1.64435	0.00015	0.02186
ENSOARG00000010283	MX1	59.0945	37.5668	-0.65357	-1.97096	5.00E-05	0.00913

Gene id	Gene	Restricted fpkm	Maintenance fpkm	log2(fold change)	Test stat	p value	q value
ENSOARG00000006257	MYBPC3	30.4213	20.9249	-0.53986	-1.57483	0.0004	0.04393
ENSOARG00000018916	MYH15	6.86614	3.7088	-0.88855	-2.0572	5.00E-05	0.00913
ENSOARG00000016029	NETO2	40.491	25.4291	-0.67112	-2.17581	5.00E-05	0.00913
ENSOARG00000018834	NLRC5	4.70296	2.17149	-1.11489	-2.0951	5.00E-05	0.00913
ENSOARG00000018472	NOBOX	21.7722	10.3635	-1.07098	-3.50804	5.00E-05	0.00913
ENSOARG00000004210	NOS1	9.64095	4.72687	-1.02829	-2.15454	5.00E-05	0.00913
ENSOARG00000010990	OOEP	17.7975	11.4802	-0.63254	-2.27774	0.0001	0.016123
ENSOARG00000010997	PADI6	96.6276	45.7188	-1.07965	-2.99871	5.00E-05	0.00913
ENSOARG00000019777	PARP2	20.5256	14.1422	-0.53742	-2.13645	0.0003	0.034708
ENSOARG00000006333	PCBD1	227.6	143.79	-0.66254	-1.70811	0.0001	0.016123
ENSOARG00000005330	PGS1	91.7791	62.5533	-0.55308	-1.60916	0.0003	0.034708
ENSOARG00000019841	PHTF1	16.4991	9.52387	-0.79276	-2.9265	5.00E-05	0.00913
ENSOARG00000004726	PLG	0.387388	1.81374	2.22712	1.76747	0.00015	0.02186
ENSOARG00000015895	PLXNC1	9.13778	5.68209	-0.68542	-1.65533	0.00045	0.047036
ENSOARG00000004585	PNLDC1	42.3089	21.5407	-0.9739	-2.59118	5.00E-05	0.00913
ENSOARG00000017730	POLDIP2	285.529	198.278	-0.52611	-1.59421	0.00015	0.02186
ENSOARG00000001660	PRELP	106.415	155.312	0.545462	1.66136	0.00025	0.031314
ENSOARG00000009728	PTN	2467.97	1536.09	-0.68406	-1.8156	5.00E-05	0.00913
ENSOARG00000012973	RALB	125.609	79.5862	-0.65835	-1.81456	5.00E-05	0.00913
ENSOARG00000010208	RBM20	3.80177	1.67841	-1.17958	-1.76804	5.00E-05	0.00913
ENSOARG00000008453	RIBC1	40.8603	23.0327	-0.82701	-1.89797	5.00E-05	0.00913
ENSOARG00000007850	ROR2	18.9176	29.7259	0.651989	1.78168	5.00E-05	0.00913
ENSOARG00000006329	RP9	40.733	64.3408	0.659536	1.83441	5.00E-05	0.00913
ENSOARG00000011291	RPL13	165.65	312.035	0.913569	2.52034	5.00E-05	0.00913
ENSOARG00000013272	RPL31	3521.58	5316.72	0.594315	1.54689	0.00025	0.031314
ENSOARG00000019941	RYR3	1.96152	0.907717	-1.11166	-2.28551	5.00E-05	0.00913
ENSOARG00000001249	SCD5	55.6473	106.013	0.92986	2.45427	5.00E-05	0.00913
ENSOARG00000004852	SCN1A	4.84708	2.73424	-0.82597	-1.96005	5.00E-05	0.00913
ENSOARG00000004415	SEMA3G	17.4536	8.78345	-0.99067	-2.07313	5.00E-05	0.00913
ENSOARG00000014882	SERPINA1	13.4193	57.4378	2.09769	4.07701	5.00E-05	0.00913
ENSOARG00000021024	SLC12A1	7.11233	3.43632	-1.04946	-2.20238	5.00E-05	0.00913

Gene id	Gene	Restricted fpkm	Maintenance fpkm	log2(fold change)	Test stat	p value	q value
ENSOARG00000018749	SLC12A3	3.41004	1.16085	-1.55461	-1.66723	0.00045	0.047036
ENSOARG00000009523	SLC25A15	37.0628	22.5169	-0.71896	-2.57932	5.00E-05	0.00913
ENSOARG00000020471	SLC2A1	25.2854	39.6862	0.65033	1.78035	0.0004	0.04393
ENSOARG00000019186	SLC5A6	30.1794	17.8977	-0.75379	-2.0361	5.00E-05	0.00913
ENSOARG00000003389	SLC7A6	49.9783	34.0024	-0.55567	-1.54455	0.00045	0.047036
ENSOARG00000008875	SLC8B1	28.3019	15.0924	-0.90708	-2.13119	5.00E-05	0.00913
ENSOARG00000007202	SPON1	113.046	184.991	0.710539	2.00034	5.00E-05	0.00913
ENSOARG00000020448	SPTBN5	2.35738	1.07548	-1.1322	-2.04303	5.00E-05	0.00913
ENSOARG00000002772	TDRD10	49.8906	21.8189	-1.19319	-2.71706	5.00E-05	0.00913
ENSOARG00000019474	TDRD15	18.1475	9.69982	-0.90374	-3.345	5.00E-05	0.00913
ENSOARG00000021052	TDRKH	47.1024	29.3589	-0.68201	-2.70108	5.00E-05	0.00913
ENSOARG00000004216	TFB1M	52.1608	32.9568	-0.66239	-1.61389	0.00025	0.031314
ENSOARG00000016713	TKT	97.4171	141.647	0.540053	1.7033	0.0002	0.027064
ENSOARG00000002868	TMC4	23.2602	13.8031	-0.75288	-1.84753	5.00E-05	0.00913
ENSOARG00000016633	TMED2	268.158	135.889	-0.98065	-2.77942	5.00E-05	0.00913
ENSOARG00000013455	TSKS	9.38945	4.32438	-1.11855	-1.66341	0.00045	0.047036
ENSOARG00000017195	TTN	2.48797	1.62481	-0.6147	-1.88397	5.00E-05	0.00913
ENSOARG00000002800	UBE2Q1	307.867	214.703	-0.51997	-1.59699	0.0003	0.034708
ENSOARG00000020918	UNC13C	1.61475	0.813592	-0.98893	-1.98386	0.0004	0.04393
ENSOARG00000009004	UPF3A	33.0526	20.5359	-0.68661	-1.71926	0.00015	0.02186
ENSOARG00000005507	WDR87	22.0535	13.3182	-0.72761	-2.36442	5.00E-05	0.00913
ENSOARG00000003121	WIF1	58.8667	37.1015	-0.66597	-1.77155	5.00E-05	0.00913
ENSOARG00000011562	YBX2	65.566	37.3915	-0.81024	-1.81632	5.00E-05	0.00913
ENSOARG00000011296	ZNF541	27.5026	18.3756	-0.58178	-1.66929	0.00015	0.02186
ENSOARG00000013902	ZP3	60.9739	29.8813	-1.02895	-2.69241	5.00E-05	0.00913

Appendix J. Conference presentations

Next Generation Sequencing Conference. 2015 Palmerston North, NZ

Application of Next Generation Sequencing in Determining Trans- generational Effects of Gestational Under-nutrition

Peter Smith[1,2], Jennifer Juengel[1], Christy Rand[2] and Jo Ann Stanton[2]

1. AgResearch Invermay, Mosgiel

2. Department of Anatomy, University of Otago, Dunedin

Developmental origins of health is of increasing interest. Negative effects of gestational under-nutrition on reproductive performance within the ensuing generation has been studied in a number of species. In this study sheep were fed either a maintenance diet or a diet 0.6 of maintenance for the first 55 days of gestation. Female fetuses were collected at gestational days 55 and 75. Remaining animals progressed to lambing and the reproductive performance of the offspring monitored through their first two breeding seasons to assess the impacts of maternal gestational under-nutrition. A transcriptome analysis using the Ion Proton is being undertaken to establish whether differential gene expression is observed in the fetal ovaries.

Society for the Study of Reproduction 2015. San Juan, Puerto Rico.

Effects of Early Gestational Undernutrition on Lamb Performance and Post Natal Fertility in Sheep

Peter Smith[1,2], Jo-Ann Stanton[2] and Jennifer L Juengel[1]. 1. AgResearch Invermay, Mosgiel, New Zealand: 2.Department of Anatomy, University of Otago, Dunedin, New Zealand

The impact of nutritional restriction during gestation on the fetus varies depending on the species, timing, severity and type of the restriction. In this study, sheep were placed on a maintenance diet, based on the metabolisable energy intake, or a diet equivalent to 60 % of maintenance for the first 55 days of gestation. Maternal blood samples were collected at gestational days 55 (n=36) and 75 (n=15). These samples were assayed for leptin and progesterone. Fetuses from these animals were also collected at gestational days 55 (n=34) and 75 (n=25). Birth weight and weaning weight of male and female lambs were recorded (n=65) from animals brought to term within each diet group. Age at puberty was determined through weekly monitoring of marking by a harnessed vasectomized ram. The ovaries of female offspring (n=30) were assessed at 6-7 months of age by laparoscopy and transrectal ultrasound scanning to determine ovulation rates and antral follicle counts; two indicators of fertility. During the first 55 days of gestation, maintenance ewes increased body weight by 4.2 % while body weight decreased 6.4 % in those on the restricted diet. Differences in body weights were maintained between the groups until at least day 90 of gestation. At both days 55 and 75 of gestation, no nutrition induced differences in fetal weight or fetal gonad weight were observed in either male or female fetuses. At cessation of nutrition restriction (day 55), maternal plasma leptin levels were significantly lower in those animals exposed to reduced nutrition (maintenance 2.9 ng/ml vs restricted 2.2 ng/ml, P=0.048). By gestational day 75 maternal leptin levels in restricted animals had returned to levels comparable to those found in maintenance animals (maintenance 3.4 ng/ml vs restricted 2.9 ng/ml). Progesterone concentrations were increased in restricted animals at gestational day 55 (maintenance 6.68 ng/ml vs restricted 7.78 ng/ml, P=0.021) while levels at day 75 were similar (maintenance 9.51 ng/ml vs restricted 9.03 ng/ml). At birth, there were no differences between the groups in the number of lambs born per ewe (maintenance = 1.77, restricted =1.73) or in the weights of those lambs born (maintenance = 5.4 kg vs restricted =5.7 kg). Growth of the lambs to weaning was also unaffected. Weights of female lambs remained similar between the groups until at least 7 months of age. The gestational nutrition restriction did not affect the onset of puberty in female offspring with the average time taken to attain puberty being 225 days for maintenance animals vs 226 days for

those from the restricted group. At 6-7 months of age, no significant differences were noted between the groups in either ovulation rate (maintenance 1.40 vs restricted 1.48) or antral follicle counts (maintenance 14.3 vs restricted 15.5). While further studies are ongoing with this cohort of sheep, the nutritional regime used here, 60 % of maintenance for the first 55 days of gestation, did not appear to affect birth rates, size or growth of offspring or peripubertal fertility of female offspring.

Society for the Study of Reproduction. San Diego, USA 2016.

Changes in Gestational Nutrition can Positively Affect Post Natal Indicators of Fertility.

Peter Smith[1,2], Christy Rand[2], Laurel Quirke[1], Jennifer Juengel[1], and Jo-Ann Stanton[2]
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While many studies report negative impacts on fertility following gestational under nutrition, we describe positive effects on indicators of female fertility in offspring following nutritional restriction of the dam during early pregnancy. Ewes (n=79) were housed indoors and fed either a maintenance diet (n=39) or a diet equivalent to 0.6 of maintenance (n=40) for the first 55 days of gestation. At day 55 animals were returned to pasture provided ad-lib. Female fetuses were recovered at day 55 (6 per group), and day 75 of gestation (6 per group) with one ovary processed for histology and one used for RNAseq analysis. Remaining animals (37) progressed to lambing, and female offspring (17 maintenance, 13 restricted) were monitored through to their second breeding season (approximately 2.5 years of age). Progesterone, FSH and LH concentrations were measured by radioimmunoassay. Statistical procedures were applied using the Genstat software package. Over the 55 days of gestation, restricted animals lost 5kg of body weight while maintenance animals gained 2kg ($p<0.01$). From 55-75 days, restricted animals gained 6.5kg and maintenance animals gained 2.8kg ($p<0.01$). At two years of age ultrasonography showed ewes from restricted dams had increased antral follicle counts (9.6 vs 6.8, $p<0.05$) and increased ovulation rates (2.0 vs 1.6, $p<0.05$). Further, these animals also showed increased concentrations of progesterone during the luteal phase ($p<0.05$). While no overall effect of group was observed on FSH concentrations, a significant group x day of cycle interaction was noted ($p<0.05$). Nutritional group did not affect LH pulse characteristics. Stereological analysis of fetal ovaries did not detect differences in germ cell numbers at day 55 of gestation. However, at day 75, fetuses from restricted dams had significantly more germ cells compared to controls (1,122,285 vs 766,344, $p<0.05$). RNAseq analysis of fetal ovaries collected on day 55 of gestation showed 100 differentially expressed ($q<0.05$) genes, of which 33 were down regulated and 67 were up regulated. At day 75, 145 genes were differentially expressed ($q<0.05$) of which 50 were down regulated and 95 were up regulated. At day 55 Ingenuity Pathway Analysis (IPA, Qiagen) identified the top functions affected as being lipid release, gonadogenesis and cell generation. At day 75, IPA identified the top functions affected as being quantity of oocytes, gonadogenesis and cell death. At both day 55 and 75 of gestation differentially expressed genes of particular interest included the germ cell specific genes

MATER, *NOBOX*, *PADI6*, and *ZP3*. Additionally at day 75 *FIGLA* and *MAEL* were also differentially expressed. These data indicate that manipulating gestational nutrition has the potential to increase fertility in female offspring. Additionally, the data is consistent with the hypothesis that the underlying mechanisms responsible for the effects of gestational nutrition on post-natal fertility are associated with perturbations to ovarian development.

Appendix K. Manuscripts in preparation

Manuscript 1. Submit to either Journal of Endocrinology or Biology of Reproduction

Increased indicators of fertility in mature female sheep following changes to nutrition during gestation

Abstract

The relationship between maternal under-nutrition and reduced post-natal fertility in female offspring has been the subject of numerous studies and reviews. Results vary markedly between studies, dependent on timing and severity of nutrition restriction, and the species studied. It is clear however, given the right conditions, that maternal under-nutrition can negatively impact the fertility of female offspring. In this study female sheep (n=40) were housed indoors and fed a diet equivalent to 0.6 of maintenance for 55 days from the day of mating. Under the same conditions control animals were fed a maintenance diet. At day 55 of gestation all animals were moved to pasture and fed ad-lib. Fetal ovaries were recovered at day 55 and 75 of gestation. Stereology was used to quantify germ cells in these ovaries and IHC used to determines germ cell proliferation, apoptosis and autophagy. Offspring (13 restricted and 17 maintenance animals) were monitored until 19 months of age. Hormone and metabolic profiles were generated for maternal ewes and fetuses at day 75 of gestation. Antral follicle counts (AFC) and ovulation rates (OR) were recorded in the peri-pubertal period (8 months of age), and in mature ewes (19 months of age) where hormone profiles were also generated. Hormone and metabolic profiles were generated for maternal ewes and day 75 fetuses.

Maternal ewes exposed showed elevated progesterone levels throughout the restricted period. No differences were observed in maternal leptin or steroid concentrations. At day 55 of gestation restricted maternal ewes showed elevated levels of albumin and Ca^{2+}, but lower creatinine levels at both days 55 and 75.No differences were observed in the metabolic factors measured in fetal plasma at day 75. Other than maternal progesterone concentrations there were no differences in either maternal or day 75 fetal plasma levels of steroids determined using LCMS. Day 75 fetal ovaries exposed to restricted nutrition contained more germ cells than control ovaries, but a lower proliferation rate (IHC using Ki-67) was observed. There were no differences observed in the levels of germ cell apoptosis (determined by TUNEL staining) or autophagy (IHC using MAPLC3) at either day 55 or day 75. Surprisingly, female offspring at 19 months of age, but not 8 months of age, showed increases in key indicators of fertility:

ovulation rate ($p < 0.05$) and antral follicle count (AFC, $p < 0.01$). Additionally, these animals showed an increase in plasma progesterone concentration ($p < 0.05$) and changes to the pattern of FSH secretion ($p < 0.05$). We propose that changes to gestational nutrition can increase indicators of fertility in female offspring and that this may be the result of the change from restricted to ad-lib feeding at a critical developmental period and not as a result of restricted nutrition alone.

Manuscript 2. Submit to BMC Genomics

Gene expression in sheep fetal ovaries exposed to changes in gestational nutrition

Abstract

Background: Numerous studies have shown the given the right conditions gestational under-nutrition can have negative impacts on the fertility of the female offspring. The cohort of animals used in the current study produced female offspring with higher indicators of fertility and morphological changes to fetal ovarian development at day 75 of gestation. While fetal germ cell development have been implicated as a process likely to be involved in the gestational nutrition post-natal fertility relationship, the mechanism(s) underlying this relationship have yet to be clearly established.

Results: Female sheep (n=40) were housed indoors and fed a diet equivalent to 0.6 of maintenance for 55 days from the day of mating (control animals were fed a maintenance diet). We used RNAseq and CuffDiff to differential gene expression in fetal ovaries recovered at day 55 and 75 of gestation. Of 15,600+ sequences RNAseq reported at day 55, 69 were differentially expressed in fetal ovaries at day 55, and 145 sequences at day 75. Fold changes observed at day 75 were less than those observed at day 55. Amongst differentially expressed genes, germ cell specific genes were prominent at both ages. Prominent Gene Ontology categories at both ages were ion transport and protease inhibitors. Pathways identified as affected using IPA analysis included some related to the arginine to nitric oxide and citrulline reaction, LXR/RXR and FXR/RXR activation, quantity of germ cells, GADD45 signalling, and acute phase response signalling.

Conclusion: The data shows that alterations to gestational nutrition alters the transcriptome of the fetal ovary. Further, differential expression of germ cell specific genes reinforce the concept that germ cell development is a major contributor to the gestational nutrition post-natal fertility relationship. Finally while the precise mechanism remains unresolved the data implicates both nitric oxide and/or protease inhibitors as potential underlying mechanisms.

Appendix L. Grants/Awards received

2015. Humphrey Russell Grant

To assist with specific project costs

2015. Student award from NGS conference

To assist with travel costs

2016. Larry Ewing Memorial Travel Award

From SSR to assist with travels costs associated with 2016 SSR conference